Abstracts of the 18th International Congress on Fibrinolysis and Proteolysis

Proteolysis in the Postgenomic Era

August 27-31, 2006
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Lipoprotein(a) Isoform Size Heterogeneity: Functional Implications for Plasminogen Activation
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Characterization of an Extracellular Proteolytic Signaling Pathway in Cancer

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Characterization of proteolytic enzymes and their substrates presents a formidable challenge in the context of biological systems. Despite the fact that an estimated 2% of the human genome codes for proteases, only a small fraction of these enzymes have well-characterized functions. Much of the difficulty in understanding protease biology is a direct result of the complexity of regulation, localization and activation exhibited by this class of enzymes. To derive signaling relationships between molecules involved in proteolytic pathways we have used various high-throughput transcriptional, translational and substrate specificity profiling methods. These approaches have been applied to members of a class of membrane-associated serine proteases (MTSP/TTSP) that are dysregulated in various cancers and to members of the granzymes that are involved in the induction of targeted cell death. Expression of several members of the MTSP/TTSP family is dysregulated in various cancers. MT-SP1 has been shown to be upregulated in certain cancers and its expression has been shown to be critical for early development in mouse models. Mild overexpression of the gene by others in a targeted manner has shown it to be sufficient for spontaneous tumor formation. Several biological substrates of MT-SP1 have been identified in vitro, and these have included molecules important for cell growth, signaling and adhesion. The membrane localization of MT-SP1 and its expression in cancer cells situates it in a unique position to participate in the mediation of oncogenic signaling. Data will be presented to show that an MT-SP1 mediated signaling pathway is co-opted in several human cancers. Correlations were determined between MT-SP1 and proposed substrates in normal and cancer tissues. Proposed substrates were also selected for the presence of characteristic MT-SP1 cleavage consensus sequences defined by functional screening of the enzyme with a combinatorial substrate library. The demonstration of MT-SP1 as the activator of a signaling pathway validates a simple and widely applicable method for the combination of biochemical functional characterization of an enzyme with a transcriptional coregulation analysis to yield new potential enzyme substrates. In a separate but related study, a cDNA expression and cleavage screen will be described that was used to identify several new substrates of granzyme B. These novel substrates reveal a previously unidentified category of granzyme B substrates whose proteolysis may be a way in which granzyme B isolates the target cell from outside survival signals. The methodologies described will hopefully shed light on the various roles that the enzymes play in functional signaling pathways.

Keywords: membrane associated serine pro, targeted cell death, cancer
The intriguing side of the hemostatic balance

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Through the intense efforts of many dedicated investigators, essentially all of the known coagulation and fibrinolytic system components (including proteases, co-factors, receptors, inhibitors and substrates) have been genetically disrupted or functionally-modified in gene-targeted mice. Detailed studies of mutant lines have dramatically improved and extended our understanding of the role of these proteins in complex physiological and pathological processes in vivo. While hemostatic derangements were the most predictable consequence of genetically-imposed deficits in key coagulation and fibrinolytic factors, sophisticated analyses in gene–targeted mice have forced many revisions in prevailing views of thrombus formation, stability and resolution. However, gene–targeted mice have revealed (or greatly amplified) another intriguing side of the hemostatic balance: many coagulation and fibrinolytic factors appear to figure prominently into a wide spectrum of biological processes in vivo that have little or nothing to do with traditional hemostasis. An early illustration of this concept was the finding that multiple factors that participate in thrombin generation and thrombin–mediated proteolysis/signaling appear to be critical for early embryonic development. Studies of mice with hemostatic deficits compatible with survival to adulthood (e.g., fibrinogen, plasminogen activator and plasminogen deficiency) have been perhaps even more revealing, illustrating an important contribution of these factors in phenomenally diverse biological contexts (e.g., tissue repair/reorganization, immune surveillance, neurodegeneration, tumor metastasis, multiple sclerosis and inflammatory diseases of the lung, kidney and joint). Regulatory crosstalk between the hemostatic and inflammatory systems has become an increasingly common theme. Our recent studies of mice with functional alterations in either clotting function or leukocyte integrin binding motifs strongly infer that leukocyte engagement of fibrin within challenged tissues may be important in leukocyte target recognition and ultimately in the implementation of specialized immune functions. A working hypothesis that has begun to emerge is that fibrin matrices within tissues may serve as a universal cue of “local danger” or “local damage” to leukocytes arriving at sites of infection or injury. The precise mechanisms by which thrombin, fibrinogen and other hemostatic factors contribute to the inflammatory response remains to be fully defined, but leukocyte–fibrin interactions through the integrin receptor Mac–1 is likely to be one important factor. More detailed studies of inflammatory processes in mice with functional alterations in hemostatic factors may reveal novel therapeutic strategies for the treatment of inflammatory diseases.

Keywords: Gene–targeting, Hemostatic factors, Inflammatory disease
Structural aspects of urokinase-type plasminogen activator receptor (uPAR) function

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The urokinase receptor (uPAR) is a glycolipid-anchored membrane protein that focalize urokinase-mediated plasminogen activation to the cell-surface through a high-affinity binding site for the growth-factor like module of uPA. This reaction assists in controlling pericellular plasminogen activation and is likely to contribute to the degradation of extracellular matrix during invasive processes. In keeping with such a role, uPAR is expressed at the invasive areas in many human cancers and a high plasma level of this receptor in these patients is a predictor of poor prognosis. Another well-defined uPAR-ligand is matrix deposited vitronectin, where the N-terminal somatomedin B domain of vitronectin plays a central role. uPAR is a modular protein containing 3 related extracellular domains, which have been assigned to the Ly-6/uPAR/a-neurotoxin protein domain family by homology considerations. Interestingly, uPAR is one of the few members of this family having multiple repeats of this domain and combined with the observation that there is a strict requirement for maintenance of the intact, three-domain structure of uPAR to express high ligand binding affinity this emphasises the importance of determining the three-dimensional structure of this receptor. Recently, the three-dimensional structures of uPAR in complex with either a peptide antagonist or the amino-terminal fragment (ATF) of the cognate ligand uPA have been solved by X-ray crystallography. These structures clearly show that each of the 3 domains of uPAR indeed adopt the predicted three-finger fold of the alpha-neurotoxins. Importantly, the modular assembly of the three domains creates a deep central ligand-binding cavity that is occupied by either of the ligands in the structures solved for the two uPAR-complexes. This cavity represents an obvious target-site for the design of small molecules interfering with cell-surface associated plasminogen activation. Alanine-scanning mutagenesis further substantiates the importance of this cavity for uPA binding, but also highlights a distinct region outside this cavity for the uPAR–vitronectin interaction, which is in accordance with the formation of a trimolecular complex between uPAR–uPA–vitronectin. These results also indicate that the SMB domain of vitronectin has reached a convergent solution for the interaction with both the serpin PAI–1 and uPAR. The recent information on the structural properties of uPAR in relation to its interactions with its biological ligands nicely illustrates how a solitary, small three-finger fold known for its cytotoxic functions has evolved into a multidomain receptor involved controlling cell surface associated plasminogen activation.

Keywords: uPAR, vitronectin, plasminogen activation
Proteolytic pathways in cell death

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The form of cell death known as apoptosis is primarily a dismantling mechanism that results in the removal of unwanted cells in vivo. The pathways that converge on the execution of this cell death program require the participation of members of the caspase family of cysteine proteases. Apoptosis is initiated by ligation of death receptors (extrinsic pathway), or developmental cues, stress and genomic damage (intrinsic pathway). These events result in the activation of apical (or initiator) caspases, and converge on the direct activation of effector (or executioner) caspases. Thus, a minimal two step activation cascade is at the heart of apoptosis. The pathways are regulated by endogenous caspase inhibitors – members of the IAP family of zinc finger proteins, which operate as direct fast binding inhibitors. Determining how apoptosis is controlled affords the opportunity to understand the fundamental processes that regulate proteolytic pathways. This talk will concentrate on biochemical and structural elucidations of caspase activation and inhibition, cast in the context of apoptosis regulation, and conclude with recent advances in the determination of the in vivo substrates of specific limited proteolysis.

Keywords: apoptosis, protease, natural substrate identification
State of the Art

Proteomics

ID: 8

Mass Spectrometry Driven Biological Discovery

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Proteomics emerged from the convergence of large-scale sequencing of genomes and high sensitivity mass spectrometry. A key element has been the use of computational algorithms to compare mass spectrometry data of peptides and proteins, in particular tandem mass spectrometry data, to the sequences of an organism to identify amino acid sequences. Algorithms to compare sequences to spectra and determine closeness of fit have enabled large-scale experiments by simplifying and automating data analysis. Simplification of data analysis unleashed the power for tandem mass spectrometers for mixture analysis. By combining high resolution separation techniques such as 1 or 2-dimensional HPLC with tandem mass spectrometry complex proteolytically digested protein mixtures can be analyzed. This approach to protein biochemistry has been termed “shotgun proteomics” when applied to mixtures of proteins. By using these techniques protein complexes, organelles, cells and tissues have been analyzed providing a new analytical paradigm to study biological systems.

Keywords: Proteomics, Mass Spectrometry, MudPIT
Activity-based proteomics of proteases in human disease

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The field of proteomics aims to characterize dynamics in protein function on a global scale. However, several classes of enzymes are regulated by posttranslational mechanisms, limiting the utility of conventional proteomics techniques for the characterization of these proteins. Our research group has initiated a program aimed at generating chemical probes that interrogate the state of enzyme active sites in whole proteomes, thereby facilitating the simultaneous activity-based profiling of many enzymes in samples of high complexity. Progress towards the generation and utilization of active site-directed chemical probes for the proteomic characterization of several classes of proteases will be described. These proteases fall into two general categories: 1) enzymes for which active site-directed affinity agents have been defined, and 2) enzymes for which active site-directed affinity agents are lacking. The application of activity-based protein profiling to the functional characterization of protease activities in models of human cancer and primary tumor specimens will be highlighted, as will be the use of this strategy as a screen to discover potent and selective reversible enzyme inhibitors.

Keywords: protease, activity-based probes, proteomics

C. Overall

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To discover novel substrates in vivo the best system-wide approach is to quantitatively identify the cleaved substrate degradome. CLIP-TAILS (Terminal Affinity Isotope Labelling of Substrates) is a new proteomic approach we have developed to identify cleaved neo-termini of substrates after enrichment. MS/MS both identifies the substrate and sequence of the cleavage site in the same experiment. Protein quantitation and identification by CLIP-TAILS labelling and MS/MS was used for substrate discovery in complex cellular contexts with the identification and validation of a myriad of new natural substrates for various MMPs. However, the simplicity of CLIP-TAILS renders it generally adaptable to other classes of protease for exploring proteolytic function in complex dynamic biological contexts. In another quantitative proteomic approach protein shedding and degradation were assayed by quantitatively determining the levels of shed proteins and protein ectodomains in the cell membrane and conditioned medium in protease transfectants. To distinguish between indirect effects of proteolysis and cleavage of substrates iTRAQ labelling and MD–LC MS/MS was used to multiplex 4 analyses in the same experiment. Thus, the effects of MMP-2 on the proteome were determined at 3, 24 and 48h. Although the approach does not directly give information on the cleavage site, because multiple peptides are identified per substrate, identification and quantitation is at a higher confidence. By mapping the location of the peptides with their abundance ratios the location of the cleavage site and domain shed can be accurately predicted by a process we term “Peptide Mapping”. The indirect effects of proteolysis can also be determined by multiplex iTRAQ experiments, such as effects wrought by alterations in the signalling environment due to proteolysis. Indeed, to truly understand the biological role of proteases it is necessary to globally integrate the identification of all the elements of the protease web—the proteases, inhibitors, cofactors and receptors—with the analysis of their interrelationships and substrates. Hence, it is critical to know in a cell or tissue the expressed protease repertoire, termed the protease degradome (Lopez–Otín & Overall, 2002 Nature Rev Mol Cell Biol 3, 509–519), all substrates of a protease—the substrate degradome—and whether they are cleaved in complex biological milieus. By analysing the direct and indirect effects of proteolytic processing on other proteases and inhibitors we are mapping the protease web. Upon network perturbation the emergent properties arising from the topology of the protease web illuminate web oscillations, robustness and recovery—essential insight into tissue and cell homeostasis in physiological and pathological circumstances. Interconnections between MMPs and other protease families found by these novel degradomic approaches reveals unexpected movement of information between all protease classes with perturbations to protease activity sending effect ripples throughout the protease web.
uPA and PAI-1: Clinically Validated Biomarkers in Breast Cancer

M. Duffy

St Vincent’s University, Dublin, Ireland

uPA and PAI–1: Clinically Validated Biomarkers in Breast Cancer, M. J. Duffy, Department of Pathology and Laboratory Medicine, St Vincent’s University Hospital, Dublin. For optimum management of patients with cancer, accurate prognostic and predictive markers are required. The primary determinant of outcome in patients with malignancy is tumor progression, especially the formation of distant metastases. Based on data from model systems, urokinase plasminogen activator (uPA) is one of the critical mediators of this process. uPA appears to mediate progression via multiple mechanisms including remodelling of the extracellular matrix, enhancing cell proliferation and migration and modulating cell adhesion. PAI–1, although originally identified as an inhibitor of uPA, is also causally involved in cancer progression. PAI–1 appears to enhance metatasis by modulating adhesion and migration. Consistent with their roles in cancer progression, multiple independent studies have shown that elevated levels of uPA and PAI–1 predict poor outcome in patients with breast cancer. The prognostic value of uPA and PAI–1 is strong (eg, RR > 2.0), independent of standard prognostic factors and found in both lymph node–negative and lymph node–positive disease. Importantly, the prognostic impact of uPA/PAI–1 has been validated in both a randomized prospective trial and a pooled analysis of 18 independent data sets (N > 8000), i.e., in 2 separate level I evidence studies. In addition to clinical validation, specific ELISAa for uPA and PAI–1 have undergone technical validation including validation in an external quality assurance program. uPA and PAI–1 are thus now ready for clinical application, especially in the identification of newly diagnosed breast cancer patients that may be able to avoid having to receive adjuvant chemotherapy. As well as supplying prognostic information, emerging data suggests that uPA and PAI–1 can also predict response to specific therapies in patients with breast cancer. Finally, clinically trials are currently underway evaluating uPA as a target for anti–cancer treatment.

Keywords: cancer, prognosis, uPA, PAI–1
Tumor Microenvironment and Proteolysis

B. Sloane

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Proteases from tumor-associated cells (e.g., fibroblasts, immune, inflammatory and endothelial cells) as well as tumor cells contribute to proteolytic pathways critical to neoplastic progression. Elevated expression (transcripts and protein) of proteases and in some cases protease inhibitors has been documented in many tumors. Nonetheless, in order to determine whether proteases play causal roles in neoplastic progression, we need techniques to measure protease activity and inhibition of that activity rather than simply the protein levels of proteases and their activators/inhibitors. As one approach, we are using confocal multi-photon microscopy to visualize degradation of quenched-fluorescent extracellular matrix proteins in real-time by live cells in 3D monotypic and organotypic cultures. In this way, we are able to both image and localize the proteolysis in 4D. Tumor cells plated as single cells self-assemble into 3D spheroids and, in so doing, exhibit pericellular proteolysis, including around single cells moving through the Matrigel. Pericellular proteolysis increases as fibroblasts are incorporated into the tumor spheroids, whereas intracellular proteolysis is observed in macrophages associated with the tumor spheroids. Using similar techniques we have observed that pericellular proteolysis accompanies the movement of single endothelial cells as they migrate together to form tubular structures, a process that takes place over a 20 hour period. The intracellular proteolysis that is observed requires functional microtubules, consistent with the matrix proteins being taken up by endocytosis, and colocalizes with markers for lysosomes. To evaluate the activity of lysosomal proteases intracellularly, we have used a selective, cell-permeable quenched activity-based probe to image activity of cysteine cathepsins. This probe reveals cysteine cathepsin activity in real-time in the live cells. We suggest that 4D imaging, along with reagents or molecular techniques to modulate protease expression/activity, will allow us to follow and define proteolytic pathways mediated by interactions of tumor cells with other cells that comprise the tumor microenvironment.

Keywords: imaging, tumor–stromal interactions, protease activity
Proangiogenic Function of Plasminogen Activator Inhibitor-1 (PAI-1) in Angiogenesis: PAI-1 Protects Endothelial Cells from FasL-induced Apoptosis

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PAI-1 plays a paradoxical positive role in tumor progression by its proangiogenic activity. We have previously reported higher levels of stromal-derived PAI-1 in more advanced human neuroblastoma tumors (Sugiura et al., Cancer Res 59:1327-36, 1999). In this study we used a human neuroblastoma orthotopic xeno-transplantation model to elucidate the mechanism involved in the proangiogenic activity of host-derived PAI-1. We observed an 80% reduction in tumor volume 4 and 5 weeks after implantation of human SK-N-BE(2) neuroblastoma cells in the adrenal gland of PAI-1\(^{-/-}\)/Rag1\(^{-/-}\) mice compared with PAI-1\(^{+/+}\)/Rag1\(^{-/-}\) mice. Tumor angiogenesis as demonstrated by angiographies and vessel quantification was significantly reduced in PAI-1\(^{-/-}\) mice. Tumors generated in PAI-1\(^{-/-}\) mice showed a decreased number of proliferative tumor cells (20% ± 1.9 BrdU positive cells in PAI-1\(^{-/-}\)/Rag1\(^{-/-}\) mice compared with 43% ± 1.7 in PAI-1\(^{+/+}\)/Rag1\(^{-/-}\) at 5 weeks) and an increase in apoptotic endothelial cells (11.9% ± 0.4 versus 4.1 ± 0.4 at 3 weeks). To understand the mechanism underlying the deficit in angiogenesis associated with the absence of host-derived PAI-1, we examined the effect of PAI-1 downregulation in human brain microvascular endothelial cells (HBMEC) by siRNA strategy. We observed that suppression of PAI-1 expression in HBMEC transfected with a PAI-1 siRNA decreased tube formation in Matrigel® by 70%. Furthermore the downregulation of PAI-1 expression resulted in a 4.7 fold increase in HBMEC cell apoptosis associated with an increase in caspase 3 and caspase 8 activities suggesting the involvement of the extrinsic apoptotic pathway. Downregulation of PAI-1 expression was associated with a significant increase of uPA activity and plasmin generation at the cell surface. We then documented that plasmin cleaved and solubilized FasL. Inhibition of plasmin activity by aprotinin or inhibition of its generation by an anti-uPA blocking antibody prevented FasL cleavage and protected HBMEC from undergoing apoptosis. A similar protective effect on apoptosis was observed with of a blocking anti-body against Fas receptor. These data thus demonstrate that PAI-1 exerts its antiangiogenic activity in part by preventing the solubilization of FasL by plasmin, thus protecting endothelial cells from FasL mediated apoptosis.
Plasminogen activator inhibitor-1 (PAI-1) is unique among the serpins because of its conformational and functional flexibility. It can occur in an active inhibitory form, a non-reactive latent form and a non-inhibitory substrate form. As PAI-1 is considered to be a risk factor in cardiovascular diseases, many efforts have been focussed at strategies to interfere with its inhibitory activity. One strategy to interfere with the PAI-1 inhibitory activity is the use of monoclonal antibodies (MA). A variety of MA with inhibitory properties have been raised against PAI-1. These inhibitory MA can be subdivided it at least three different categories. Firstly, MA that inhibit PAI-1 by preventing the initial formation of the Michaelis complex between PAI-1 and its cognate proteinases. Secondly, MA that induce a substrate behaviour in PAI-1 (switching MA). Thirdly, MA that accelerate the latency conversion of PAI-1. Elucidation of their epitopes, revealed six distinct regions as potential target sites to interfere with the inhibitory properties of PAI-1. Moreover, five out of six single-chain variable fragments (scFv) from the corresponding human PAI-1 neutralizing MA revealed similar affinity constants as well as similar functional effects on PAI-1 activity suggesting that even smaller molecules might be able to interfere with the PAI-1/proteinase interaction. Targeting these functional epitopes was further confirmed by introduction of a cysteine at the putative target site of one MA (located on the loop between ?-helix I and ?-strand 5A) and subsequent specific reaction with low molecular mass sulfhydryl–specific reagents. Taken together these data indicate that targeting a functional epitope in PAI-1 with a scFv or with a small synthetic compound may be a feasible strategy in rational drug design aiming at pharmacological modulation of PAI-1 activity.

Keywords: Serpins, PAI-1, drug design
Plasminogen activator inhibitor–1 (PAI–1) regulates fibrinolysis and has been reported to be an independent predictor of the development of type 2 diabetes mellitus and ischemic cardiovascular events. We have systematically investigated the role of PAI–1 in thrombosis, arteriosclerosis and atherosclerosis using genetic models of PAI–1 excess and deficiency. Transgenic mice that overexpress a stable variant of active human PAI–1 under the control of murine preproendothelin–1 promoter develop spontaneous thrombotic occlusions of coronary arteries with histological evidence of subendocardial infarction identified in the absence of hyperlipidemia or atherosclerosis. Genetic PAI–1 deficiency and administration of an orally active PAI–1 antagonist protects against the development of coronary arteriosclerosis and hepatic vein thrombosis induced by chronic nitric oxide synthase (NOS) inhibition or aortic arteriosclerosis generated by infusion of angiotensin II. Finally, PAI–1 deficiency (genetic or therapeutic) paradoxically accelerates the development of coronary atherosclerosis and promotes the development of myocardial infarction in ApoE deficient mice. These studies indicate that the chronically elevated levels of PAI–1 alone are sufficient to cause coronary arterial thrombosis in mice and that PAI–1 deficiency can protect against the structural vascular changes that accompany hypertension in the setting of long-term NOS inhibition. However, PAI–1 appears to provide an anti-inflammatory, protective benefit that retards the development of coronary atherosclerosis. These findings suggest that direct inhibition of vascular PAI–1 activity may provide a new therapeutic strategy for the prevention of arteriosclerosis and thrombotic cardiovascular events in humans. Importantly, there is likely an optimal range of PAI–1 that simultaneously reduces the risk of intravascular thrombosis while maintaining the protective, anti-inflammatory effects of PAI–1 against atherosclerosis.

Keywords: PAI–1, vascular pathology
Role of the fibrinolytic system in the development of obesity and the metabolic syndrome

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Increased circulating levels of plasminogen activator inhibitor-1 (PAI-1) belongs to the metabolic syndrome (MS) and could contribute to the associated elevated cardiovascular risk. PAI-1 promotes fibrin accumulation and is also involved in cell migration, angiogenesis and fibrosis. Thereby, PAI-1 may locally contribute to atherosclerotic vessel wall and adipose tissue remodelling. The contribution of PAI-1 to the development of obesity and the MS, begins to be established. A direct connection between PAI-1 and the action of insulin has been shown in vitro. Addition of vitronectin to fibroblasts cooperates with insulin to induce protein kinase B phosphorylation. PAI-1 is able to prevent this cooperation in a vitronectin-dependent manner. PAI-1 deficiency enhances the insulin stimulated glucose uptake by adipocytes and is able to prevent the deleterious effect of TNF on insulin sensitivity of adipocyte. In addition, PAI-1 controls adipocyte differentiation. Inhibition of PAI-1 with a neutralizing antibody or PAI-1 deficiency promotes adipocyte differentiation. Conversely, overexpression of PAI-1 by adenovirus-mediated gene transfer inhibits differentiation. Interesting data were obtained in vivo. PAI-1 deficiency as well as pharmacological inhibition of active PAI-1 improves some key biological features of the metabolic syndrome whereas under standard fat diet aged mice overexpressing murine PAI-1 exhibit higher insulin and triglyceride levels. Due to the effect of PAI-1 on adipocyte insulin sensitivity and differentiation one could expect that PAI-1 deficiency will lead to increase fat accumulation. Surprisingly two groups found that fat accumulation was prevented in mice lacking PAI-1 in two different kinds of models, a nutritionally induced and a genetic murine model of obesity. The protection against obesity was linked to an increase in metabolic rate, total energy expenditure and thermogenesis. Overall these data support the concept that PAI inhibition has the potential to reduce obesity, its complications and may represent a new interesting therapeutic target.

Keywords: PAI-1, Obesity, Metabolic syndrome
Matrix Protease Responses of the Neurovascular Unit During Ischemic Stroke

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The cerebral microvasculature is unique in ultrastructure and function. The cell–matrix inter–relationships within cerebral microvessels and the relationships among the cellular components of the neurovascular unit are significantly altered by ischemic injury. Activation of hemostasis underlies both focal cerebral ischemia and the initiation of the inflammatory response, which is in turn central to the pathogenesis of ischemic stroke. Within the ischemic core both fibrin deposition and platelet activation contribute to microvascular obstruction. While inhibition of platelet integrin alphaIIb/beta3 significantly reduces “focal no-reflow,” inhibition of the platelet–fibrin interaction augments the risk of deleterious local hemorrhage. The latter highlights the roles of protease generation initiated by focal ischemia at the microvascular endothelial cell–astrocyte interface. Species–dependent generation of pro–MMP–2 or pro–MMP–9 occurs following middle cerebral artery occlusion. In one system, pro–MMP–2 and its activation systems (MT1–MMP, MT3–MMP, and u–PA) appear concordantly within the ischemic core. pro–MMP–2 generation correlates directly with neuron injury. Significant loss of the basal lamina components collagen type IV, fibronectin, laminins, and the HSPG perlecan accompany disruption of endothelial cell– and astrocyte matrix receptor integrity. In addition, select cysteine proteases are generated within the neurovascular unit which are capable of degrading vascular HSPG. These are expressed by both target microvessels and neighboring neurons within the early ischemic period. Whether these proteases contribute directly to the increase in microvascular permeability (loss of the blood brain permeability barrier) is still uncertain. However, interventions which limit these early post–ischemic processes in the neurovascular unit can significantly reduce cerebral injury.

**Keywords:** ischemic stroke, matrix proteases, microvasculature
Matrix metalloproteinases in brain injury, hemorrhage and remodeling after stroke

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Matrix metalloproteinases (MMPs) are implicated during acute brain injury after stroke. By degrading neurovascular matrix, MMPs mediate edema and hemorrhage. By disrupting cell–matrix homeostasis, MMPs mediate neuronal anoikis. These acute events at the brain–vascular interface play critical roles in neuroinflammation as stroke progresses. MMP knockout mice are protected against focal cerebral ischemia and transient global cerebral ischemia. Signaling links between the MMP system and the plasminogen system underlie some of the complications of tPA thrombolytic therapy for acute ischemic stroke. Experimental findings may yet translate, as plasma MMPs are now beginning to be assessed as biomarkers for predicting responses to therapy. However, in contrast to acute events, MMPs play different roles during stroke recovery. By modulating matrix, MMPs may be involved in neuroblast migration from the subventricular zone. And in peri-infarct cortex, MMPs may play a role in neurovascular remodeling. Understanding these diverse actions of MMPs in damaged and recovering brain may eventually yield new targets for therapy in the acute and chronic phase after stroke.

Keywords: neurogenesis, neurovascular unit, blood–brain barrier
Neuroprotective mechanisms of activated protein C

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Thrombotic stroke is a major cause of death and disability with an estimated incidence of 700,000 cases annually in the US. Currently, tissue-plasminogen activator (tPA) is the only approved therapy for the treatment of acute ischemic stroke in the US. tPA treatment, however, is associated with a significant risk for symptomatic brain hemorrhage, neurotoxicity and a brief 3–h time window of efficacy. Thus, improved therapies for ischemic/thrombotic stroke are needed. Activated protein C (APC) is a serine protease with systemic anticoagulant, anti-inflammatory and antiapoptotic activities. We have recently reported that APC reduces brain damage after transient ischemia and embolic stroke in rodents and inhibits the intrinsic and extrinsic apoptotic pathways in injured neurons and ischemic brain endothelium. In addition to its direct neuronal protective activity, APC stabilizes vascular endothelial barriers and has a low risk for brain hemorrhage in patients with sepsis and in animal models of stroke. APC can also protect neurons and neurovascular cells from tPA toxicity and tPA-induced hemorrhage by inhibiting the tPA-matrix metalloproteinase-9 pathway in ischemic brain endothelium in vivo and in vitro. In this presentation, I will discuss neuroprotective mechanisms of APC and why we believe APC should be developed clinically as a standalone therapy for stroke and/or as a candidate for tPA adjunctive therapy. Supported HL63290.

Keywords: Activated Protein C, Stroke, Neuroprotective
Angiostatin4.5: From the Bench To The Bedside

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Angiostatin4.5 (AS4.5) is the naturally occurring human isoform of angiostatin, a potent inhibitor of angiogenesis, consisting of kringles 1–4 and 85% of kringle 5 of plasminogen. We have previously shown that plasminogen is converted to AS4.5 in a two-step reaction. First plasminogen is activated to plasmin by a plasminogen activator, followed by plasmin autoproteolysis within kringle 5. We have now shown how this reaction occurs on the surface of prostate and other cancer cells. Surface globular beta-actin serves to avidly bind kringle 5 of plasminogen (KD 140 nM). Actin bound plasminogen is converted to plasmin by urokinase (uPA) bound to the urokinase receptor (uPAR). Plasmin remains bound to surface actin with a comparable affinity as plasminogen. Subsequently, surface actin serves as a cofactor for plasmin autoproteolysis within kringle 5 to AS4.5. Following kringle 5 cleavage, the AS4.5 can no longer bind to actin and enters the systemic circulation. Thus these data help explain the paradigm that angiogenesis promoters (i.e. plasmin) act locally, while angiogenesis inhibitors (i.e. AS4.5) act systemically. In a new pilot study, we have characterized expression of uPAR and actin on the surface of normal prostate epithelium and cancer specimens. Beta-actin was present on the surface of the prostate epithelium, with similar levels in normal, low-grade cancer, and high-grade cancer. Interestingly, uPAR was expressed abundantly in normal prostate epithelium, and expression was reduced in low-grade cancers, and further reduced in high-grade cancers. These results may suggest that a reduced ability of aggressive prostate cancer cells to generate AS4.5 may explain, in part, why some cancers are more invasive and metastatic. We have also shown that plasmin autoproteolysis to AS4.5 can be mediated by a small molecule free sulfhydryl donor, in lieu of globular actin. As plasminogen activators and free sulfhydryl donors are available as FDA approved drugs, we tested the hypothesis that administration of an Angiostatic Cocktail, consisting of a plasminogen activator and a free sulfhydryl donor, to cancer patients would result in increased plasma levels of AS4.5. A phase 1 clinical trial was performed with co-administration of escalating doses of tissue plasminogen activator (1, 2, 3, 5 mg/hr of tPA) and mesna (240 mg/m2 bolus followed by an infusion of 50 mg/h). A dose–response relationship of tPA dose with increased AS4.5 levels was observed. In the 5 mg/hr tPA cohort, mean AS4.5 levels increased from a baseline of approximately 20 nM to a peak of 110 nM. Further, in 2 of the 5 patients in the 5 mg/hr tPA cohort the serum markers declined in response to 8 weeks of the Angiostatic Cocktail, although tumor mass measurements did not decline. No therapy–related toxicity was observed, although one patient in the 5 mg/hr tPA cohort paradoxically developed a pulmonary embolism. This confirms the ability to safely to generate AS4.5 in vivo by the Angiostatic Cocktail. Further studies of the Angiostatic Cocktail, including further dose escalations, are in preparation.

Keywords: angiostatin, plasminogen, angiogenesis
The role of plasminogen activator inhibitor-1 (PAI-1) in angiogenesis has been extensively studied but still remains controversial. Utilizing endothelial cells (EC) from wild-type (WT) and PAI-1 deficient (PAI-1−/−) mice, the impact of a host PAI-1 deficiency on cell proliferation, an event associated with angiogenesis, was evaluated. Initial observations demonstrated that EC from PAI-1−/− mice proliferate at an enhanced rate compared to WT cells. This phenotype in PAI-1−/− cells could be associated with hyperactivation of Akt(P-Ser473), and is likely due to enhanced inactivation of phosphatase PTEN, rendering the cells resistant to apoptotic signals. This event appeared to be PI3-κ-dependent, as the pharmacological inhibitor wortmannin not only inhibited phosphorylation of Akt, but also diminished the rate of proliferation in PAI-1−/− EC. Marked phosphorylation of the downstream target of Akt, caspase-9 at threonine163 was observed in proliferating PAI-1−/− EC rendering it inactive. This led to lower levels of pro- and cleaved-caspase-3, thus protecting the PAI-1−/− cells from caspase-mediated apoptosis. When the PAI-1 deficiency was circumvented by addition of exogenous r-PAI-1 to PAI-1−/− EC, cell proliferation was restored to that of WT levels. Furthermore, addition of r-PAI-1 concomitantly decreased the levels of Akt(P-ser473) and pro-caspase-3 in PAI-1−/− EC. Additional studies using different variants of PAI-1 demonstrated that regulation of PAI-1 is dependent on its interaction with low density lipoprotein receptor–related protein. These observations suggest that PAI-1 is a negative regulator of cell proliferation, and reveals a potential mechanism by which PAI-1 regulates the activation of the Akt pro-survival pathway resulting in altered cell proliferation and apoptosis. Studies in mice deficient for specific functional domains of PAI-1 should further elucidate mechanisms by which PAI-1 functions in these and other endothelial cell functions pertinent to the angiogenic process.

**Keywords:** PAI-1, Angiogenesis, Endothelial cells
Distinct roles for cysteine cathepsins in multistage tumorigenesis

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The aberrant tumor microenvironment is increasingly appreciated as a key determinant of cancer progression, involving multiple cell types and functional interactions. Several matrix-degrading enzymes have been found to modulate regulatory networks governing tumor progression, and thus their inhibition has the potential to block multiple tumor-promoting signals and effector functions. We have previously shown that several cysteine cathepsins are upregulated in the RIP-Tag mouse model of pancreatic islet cell carcinogenesis, and that tumor progression is impaired following their collective pharmacologic inhibition. However, inhibiting an entire family of proteolytic enzymes has the additional potential to produce unwanted effects, including toxicity. As such, determining the critical target genes within implicated protease families is particularly relevant. Therefore, we have now taken a genetic approach to identify the individual cathepsin(s) that are critically important. Interestingly, not all cathepsins are functionally equivalent in promoting carcinogenesis. Mutants of cathepsins B or S impaired tumor formation and angiogenesis, while cathepsin B or L knockouts significantly retarded cell proliferation and tumor growth. Absence of any one of these three genes impaired tumor invasion. In contrast, removal of cathepsin C had no effect on either tumor formation or progression. We have identified E-cadherin as a target substrate of cathepsins B, L and S, but not cathepsin C, potentially explaining their differential effects on tumor invasion. Furthermore, we found analogous increases in cathepsin expression in human pancreatic endocrine neoplasms, and a significant association between levels of cathepsin B and L and tumor malignancy. These genetic experiments have revealed novel roles for individual cathepsins in tumor angiogenesis, invasion, apoptosis and cell proliferation, and indicate that there are important functional differences between cathepsin family members that would not have been predicted from biochemical analyses. In addition to increasing our understanding of how cathepsins specifically contribute to tumor-host interactions in the cancer microenvironment, these results may help guide the design of clinical trials aimed to assess cathepsin inhibitors as cancer therapies.

Keywords: cancer, cysteine cathepsin, invasion
Thrombus Formation In Vivo

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The study of thrombus formation at the molecular and cellular level in a living animal offers the opportunity to reexamine the tenets and models of thrombus formation proposed on the basis of extensive in vitro and more indirect in vivo analyses. To exploit this potential, we have established an intravital videomicroscopy system that can directly visualize thrombus formation in arterioles and venules in an animal model. After vessel wall injury in the microcirculation, thrombus development can be imaged in real time. We have used this system to begin to explore the role of platelets, blood coagulation proteins, endothelium, and the vessel wall during thrombus formation in vivo in real time. The ability to study the biochemistry and cell biology of thrombus formation in living animals has begun to provide a new understanding of this complex biologic process. Although many of the constructs developed from in vitro studies are observed in vivo, intravital studies of thrombus formation have begun to reveal some important features of the thrombotic process in vivo that break with conventional wisdom. First, it is now clear that platelet activation and platelet thrombus formation are intertwined with thrombin generation and fibrin clot propagation. These pathways are temporally and spatially integrated. Second, platelet accumulation, originally thought to be exclusively dependent on vWF, involves multiple proteins, including vWF, fibrinogen, and possibly fibronectin. Third, the platelet–platelet synapse, classically described as glycoprotein IIb/IIIa interaction with fibrinogen, appears to involve numerous adhesion molecules besides glycoprotein IIb/IIIa. Fourth, blood–borne tissue factor is initially delivered to the developing thrombus in a process dependent on P–selectin and PSGL–1; leukocytes bearing tissue factor either do not have a role or have a role later in thrombus formation. Fifth, laser–induced vessel wall injury activates the tissue factor pathway to thrombin generation. This thrombosis model, which may have features similar to those characterized by inflammation, does not involve the subendothelial matrix and specifically does not involve collagen. In contrast, ferric chloride injury leads to collagen exposure in the subendothelial matrix. Exposed collagen triggers platelet activation in a mechanism mediated by glycoprotein VI. Sixth, intracellular calcium mobilization is necessary for stable platelet interaction with the thrombus. Seventh, thrombus generation is a highly complex process requiring many components, both structural and regulatory. Although there is some redundancy, elimination of any of these multiple components disrupts thrombus formation. This observation provides opportunities for targets for novel antithrombotics but also indicates how empirical it will be to identify the optimal targets. Lastly, the dogma that activated platelets provide the membrane surface for thrombin generation needs to be questioned and the critical membrane surfaces in vivo determined.

Keywords: Thrombosis, In vivo imaging
Dynamic imaging of protease location and function in ECM remodeling

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Tumor invasion and metastasis involve a cascade of proteolytic processes towards the adjacent extracellular matrix (ECM), yet the subcellular location and topographic control of ECM remodeling are unknown. Using “4D biochemistry” of HT–1080 cell invasion in 3D collagen lattices, we simultaneously visualized the location and dynamics of (i) MT1–MMP/EGFP, (ii) enzymatic substrate degradation using the dequenching of FITC-collagen or cleavage neoepitope staining, (iii) the structural remodeling of individual collagen fibers by confocal reflection microscopy, and (iv) changes in cell morphology during migration. The findings integrate pericellular proteolysis of ECM into the cell migration cycle and specify two distinct spatio-temporal types of ECM remodeling. First, during single-cell migration within a randomly organized fibrillar collagen scaffold, cleavage of individual fiber belts discretely occurs at regions of focal pressure at branching pseudopods and the region of cell body expansion, but not the anterior leading edge required for force-generation. Thus, the protruding cell front consists of two distinct zones, an anterior adhesion and a posterior proteolytic zone. Second, smooth boundaries of prealigned ECM tracks are continuously degraded by cell masses thereby slowly shifting the cell–ECM interface towards the matrix compartment. Whereas the first type generates small tracks and space for individual cell movement, the second provides large zones cleared of ECM to allow tumor expansion or the movement of cell masses and collectives along textured ECM. These findings show structurally and biochemically how tumor growth and invasion into adjacent ECM generate histologically characteristic invasive cell and tissue patterns.

Keywords: collagen remodeling, MMPs, cancer invasion
In vivo Imaging of protease activity using fluorescent activity based probes

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Protease activity is often tightly regulated in both normal and disease conditions. However, it is difficult to monitor the dynamic nature of this regulation in the context of a live cell or whole organism. Our laboratory has developed a series of fluorescent small molecule probes that covalently modify protease targets through an activity dependant chemical reaction. These reagents freely penetrate cells and can be used to directly image protease activity in live cells and whole animals. In addition these reagents can be used to monitor global patterns of protease activity in complex proteomes. Specific progress using NIRF based probes in mouse models of cancer will be discussed.

Keywords: protease, activity based probe, functional imaging
Matrix Metalloproteinases

**ID:** 264

**MATRIX REGULATION OF OVARIAN CANCER METASTASIS**

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Matrix Regulation of Ovarian Cancer Metastasis. J.E. Symowicz, M. Barbolina, Laurie Hudson & M.S. Stack, Northwestern University, Chicago, IL, USA and Univ. of New Mexico, Albuquerque, NM. Epithelial ovarian carcinoma is the leading cause of death from gynecologic malignancy and 75% of women exhibit metastases at diagnosis. Tumor nodules are exfoliated as single cells or multi–cellular aggregates and generate diffuse intraperitoneal (i.p.) metastases and malignant ascites. Following attachment to the peritoneal mesothelium, integrin–mediated cell–matrix interaction potentiates i.p. dissemination. As metastasis is the result of numerous i.p. adhesive events, cellular integrins play a major role in regulation of ovarian cancer metastatic behavior. Ovarian cancer cells adhere preferentially to interstitial collagens types I and III using a2b1 and a3b1 integrins, representing an important early event unique to ovarian cancer metastatic dissemination. Proteolytic activity is important at multiple stages in i.p. metastasis, including disruption of cell–cell junctions, migration and invasion through the mesothelial cell layer and the submesothelial basement membrane, and for subsequent tumor–mediated angiogenesis. While OSE does not express matrix metalloproteinases (MMPs), several MMPs including MMP–9 and MT1–MMP (MMP–14) are expressed in malignant ovarian tumor cells as evaluated by in situ hybridization and immunohistochemistry. To test the hypothesis that integrin–mediated adhesion to submesothelial collagen functions as a key event in ovarian tumor metastasis via modulation of MMP function, cells were cultured on 3D collagen gels or incubated with bead–immobilized integrin antibodies to induce integrin clustering. Engagement of collagen binding integrins induced rapid loss of surface E–cadherin as shown by immunofluorescence microscopy and surface labeling/western blotting. Loss of E–cadherin–associated b–catenin was also observed and was correlated with a corresponding increase in nuclear b–catenin levels and enhanced transcriptional activation of the b–catenin reporter construct TOPFLASH. Expression of known b–catenin target genes including COX2, MMP–14, and uPA as well as MMP–9 was enhanced following integrin clustering. Further, extended integrin aggregation induced shedding of an 80 kDa E–cadherin ectodomain fragment in an MMP–9–dependent manner. E–cadherin ectodomain was prevalent in ascites and sera from ovarian cancer patients as detected by ELISA and western blot. These data suggest that contact with submesothelial collagens can potentiate metastatic dissemination via proteolytic and non–proteolytic loss of cell–cell junctions. Following junction disruption, invasion of an interstitial collagen–rich matrix requires collagenolytic activity. Using real–time RT–PCR, western blotting and zymography, our results indicate an upregulation of MT1–MMP protein and mRNA on 3D relative to 2D collagen, resulting in enhanced collagen invasive activity. 3D collagen culture induced expression of the transcription factors Egr–1 and –2, and knockdown of Egr–1 using siRNA abrogated the ability of 3D collagen to induce MT1–MMP. Together these data support the hypothesis that matrix status influences ovarian cancer metastatic potential through modulation of proteinases that modify cell–cell and cell–matrix contacts.

**Keywords:** MMP, collagen, ovarian cancer
MMP-9-dependent regulation of angiogenesis and invasion in GBM

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Glioblastomas (GBM) are very angiogenic and invasive tumors. GBM infiltrate as single cells in the white matter of the brain and travel on the outside of blood vessels in the perivascular space dispersing significant distances into the brain parenchyma. Although a lot of attention has been given to the infiltrating GBM phenotype, still very little is known about why and how GBM cells move along blood vessels; a phenotype that can be identified in every GBM. We were able to switch GBMs to a nearly 100% perivascular invasion mode when we deleted the hypoxia transcription factor HIF-1α in genetically engineered glioblastomas (HIFko). These tumors were unable to induce angiogenesis, partly by recruiting less bone marrow-derived cells, but adapted by becoming extremely invasive. Interestingly, HIFko tumors showed a dramatic reduction in MMP-9 positive cells. MMP-9 was mainly expressed in a subset of infiltrating tumor cells and bone marrow–derived cells that were recruited to the tumor site. Genetic knockout analyses revealed that loss of MMP-9 in GBM leads to non-angiogenic tumors with increased perivascular invasion, similar to the phenotype observed in HIF-deficient tumors. These data suggest that MMP-9 is a critical protein in angiogenesis and infiltration of GBM but not required for perivascular tumor cell invasion.

Keywords: MMP-9, GBM, angiogenesis
Proteolytic Remodeling of the 3-Dimensional Extracellular Matrix

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During growth and development, the identity of the protease– dependent or independent systems required to support cell migration, proliferation and differentiation within the confines of the 3– dimensional (3–D) extracellular matrix have remained the subject of considerable controversy. Using a series of ex vivo and in vivo systems, however, a body of evidence has begun to accumulate to suggest that a triad of type I transmembrane matrix metalloproteinases, termed MT1–MMP, MT2–MMP and MT3–MMP, play a dominant role in controlling complex cell behaviors ranging from fibroblast migration and angiogenesis to adipocyte differentiation. Like other members of the matrix metalloproteinase gene family, the MT–MMPs are synthesized as latent enzymes, but in contrast to almost all of the secreted MMPs, the membrane–anchored enzymes undergo intracellular activation following proprotein convertase–dependent processing. Once displayed at the cell surface, MT–MMPs function as direct–acting proteinases which remodel both basement membrane– and interstitial matrix– associated macromolecules in order to drive 3–D invasion, growth and differentiation programs. While cell behavior is largely unaffected under 2–D conditions in the absence of MT–MMP activity, cell function is severely compromised within the confines of the 3–D cross–linked extracellular matrix that defines normal adult tissues. Taken together, these studies define a new and dominant role for MT–MMPs in 3–D extracellular matrix remodeling events critical to normal cell function in health and disease.

Keywords: remodeling, extracellular matrix, MMPs
A Tale of Two Plasminogen Receptors

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Plasminogen receptors (PlgRs) localize the broad proteolytic potential of plasminogen to cell surfaces, where it can facilitate matrix degradation and growth factor generation, events that promote cell migration. PlgRs fall into two categories with respect to their recognition of plasminogen: those with or those without a C-terminal lysine. Recognition of plasminogen by both categories of PlgRs can be mediated by the lysine binding sites of plasminogen. A new representative to the category of PlgRs with a C-terminal lysine is histone H2B (H2B). Like many other PlgRs, H2B does not have a signal sequence but its cell-surface expression is well documented in the literature. Human cells and cell lines, which exhibit enhanced plasminogen binding, adherence-stimulated U937 cells and cultured neutrophils, show marked upregulation of H2B surface expression. H2B contributes 40–60% of the increased plasminogen binding capacity of these cells, as assessed by the blocking effect of FAB fragments of antibodies raised to the C-terminus of H2B. A role of H2B in plasminogen binding to mouse macrophages also can be demonstrated with these C-terminal blocking FAB fragments. Furthermore, intravenous administration of the FAB fragments into mice reduces the peritoneal recruitment of mouse macrophages in response to an inflammatory stimulus. Thus, H2B represents a functionally important PlgR. Integrin alpha-M beta-2 lacks a C-terminal lysine but still binds plasminogen via its lysine binding sites. In addition, alpha-M beta-2 can also bind uPA; and, through this assembly, can markedly enhance plasminogen activation. This effect can result in enhanced fibrinolysis and cell migration as shown by the suppressive effect of alpha-M beta-2 blocking reagents. In addition to the extracellular consequences of plasminogen binding to alpha-M beta-2, this interaction can induce intracellular signaling events. Signaling can be demonstrated by the induction of tyrosine phosphorylation events upon plasminogen binding to neutrophils. Among the downstream consequences of plasminogen-induced intracellular signaling events is the protection of neutrophils from apoptosis. Together, these data indicate that H2B and alpha-M beta-2 play a significant role in cell biology via their capacity to interact with plasminogen.

Keywords: plasminogen receptors, integrins, histone H2B
Urokinase Receptor-dependent Cell Signaling

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The receptor for urokinase-type plasminogen activation (uPA) has emerged as a multifunctional regulator of cellular phenotype, which may be particularly important in cancer. By binding uPA, uPAR organizes a cascade of proteases at the cell surface, including plasmin and matrix metalloproteases, which may facilitate cellular penetration of tissue boundaries. uPAR functions in cell adhesion by binding directly to vitronectin. Furthermore, uPAR initiates cell-signaling in response to ligation with either uPA or vitronectin. The signaling pathways activated by these two ligands appear distinct, leading to activation of Rac1 and ERK/MAP kinase, respectively. Both cell-signaling pathways control different processes involved in determining the rate of cell migration. uPAR-dependent cell signaling also promotes cell survival. Because cell-signaling in response to uPA occurs even in cells with low levels of cell-surface uPAR and with low receptor fractional occupancy, autocrine signaling through uPAR may be a major determinant of the basal level of ERK/MAP kinase activation in cells in culture under serum-free medium. Recent work from our laboratory and from others suggests that uPAR-dependent cell signaling actually reflects the function of a multi-component signaling receptor complex that is dynamic in nature and may include integrins, the epidermal growth factor receptor (EGFR), other receptor tyrosine kinases, and the G protein-coupled receptor, FPRL-1. In this talk, we will present new results regarding the synergistic function of uPAR, the EGFR, uPA and EGF. We will then describe novel pathways by which uPAR-dependent cell signaling may transform the phenotype of breast cancer cells, so that these cells are more aggressive in behavior. Finally, we will present new results focusing on the function of uPAR as a signaling receptor in cancer in vivo. Our data suggest that uPAR-dependent cell signaling may be regulated within a tumor by the degree of oxygenation. Furthermore, we have new evidence supporting the hypothesis that non-malignant cells within a cancer establish paracrine pathways, in which uPA is delivered to uPAR expressed by the malignant cells. Our results will support the hypothesis that non-malignant cells within the tumor microenvironment regulate cancer invasion and metastasis, based uPA trafficking between cells.

Keywords: Urokinase Receptor, Cell Migration, Cell Survival
Protease Signaling in Angiogenesis and Cancer

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Tissue Factor (TF) has dual roles in biology, serving as the initiator of the coagulation cascade and as a signaling co-receptor that supports protease activated receptor cleavage (PAR) by associated coagulation proteases. TF serves as the anchor for two distinct signaling complexes: the binary TF-VIIa complex that cleaves PAR2 and the coagulation-initiation ternary TF-VIIa-Xa complex that activates PAR1 or 2 in dependence of Xa. These alternative signaling complexes are supported by distinct cell surface pools of TF. Our recent studies show that disulfide isomerization acts as a switch between TF-VIIa signaling and coagulation activation and concomitant ternary complex signaling. TF-VIIa signaling requires association of protein disulfide isomerase (PDI) with TF. In this pathway, PDI inhibits TF coagulant activity by breaking the extracellular TF Cys186-Cys209 disulfide in a nitric oxide-dependent redox pathway. Thereby, signaling by the TF-VIIa complex proceeds independent of coagulation activation. Tumor biology and neoangiogenesis are two major areas where TF plays important pathophysiological roles. TF can either act as a signaling receptor or support tumor cell malignant behavior through coagulation activation. Metastasis is dependent on TF-mediated thrombin generation, platelet- and fibrin-deposition, and protease activated receptor (PAR) 1-signaling. In contrast, TF-dependent signaling plays prominent roles in angiogenesis and primary tumor growth. With human TF specific inhibitory antibodies that selectively block TF-VIIa signaling or coagulant activity on tumor cells in xenograft models, we showed that TF-VIIa signaling is the primary pathway that supports tumor growth. We have further established in a retinal, hypoxia-mediated neoangiogenesis model that TF-VIIa mediated PAR2 signaling, rather than PAR1 signaling is the major TF-driven angiogenic response. These studies provide strong support for a key role of protease activated receptor signaling in driving pathology of the TF coagulation pathway.
New Thrombolytic Therapies

**ID:** 251

**Engineered Human Proteases (Alterases) as a Broad New Therapeutic Modality**

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Several recombinant proteases are currently used in the clinic, clearly establishing this class of enzymes as a potential source of safe and efficacious new biopharmaceutical agents. All of these marketed proteases mediate clinical benefit by cleaving their normal physiological or cognate substrate. The ability to target proteases selectively to new, non-cognate substrates could significantly expand the uses of proteases as drugs and may provide opportunities for protease therapeutics comparable in depth and scope to those currently available to antibody therapeutics. As initial proof of concept studies, we have used both rational design and a novel protease selection technology to redesign a wild type human protease to cleave and inactivate either a growth factor receptor (VEGFR2) or a key component of the human complement cascade (C2). We have demonstrated activity in vitro and in vivo for both of these engineered proteases that is at least comparable to the activity of a corresponding antibody that is approved (Avastin) or in phase III clinical development (pexelizumab). We also demonstrate a clear advantage of protease therapeutics compared with antibodies or small molecule therapeutics against membrane associated targets – the cleavage product created by the protease serves a direct pharmacodynamic marker, significantly facilitating both preclinical and clinical development. Engineered proteases may represent an exciting new approach to biotherapeutics with potential broad application in multiple diseases.

**Keywords:** Protease, complement, VEGF
Plasmin: A Direct Thrombolytic Agent

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Direct thrombolytic agents, of which the prototype is represented by plasmin, offer the potential for distinct clinical advantages over plasminogen activators such as tissue plasminogen activator (TPA). In addition to full-length plasmin, crude preparations of which were utilized therapeutically 50 years ago, novel mutant derivatives of plasmin (mini-, micro-, delta-plasmin) and snake venom extracts (eg, fibrolase) or recombinant products based on such extracts (alfimeprase) are at various stages of pre-clinical and clinical assessment. All of these agents share the following qualities (1,2):

- After systemic infusion: o Inactivation by high concentration of natural inhibitor present in blood. o Competitive binding to and degradation of plasma protein substrates. o Limited binding to distant vascular thrombotic occlusions. o Ineffective thrombolysis.

Keywords: Plasmin, Thrombolysis, Inhibitors
Microplasmin: A novel, direct-acting thrombolytic for treatment of acute vascular occlusions

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INTRODUCTION Microplasmin, a recombinant DNA–derived protein (Mr 27,229) is a truncated form of human plasmin (with protease domain retained). It is produced by expression of the human microplasminogen gene in the yeast Pichia pastoris, which is then cleaved and converted to microplasmin. Microplasmin is a novel thrombolytic that may possess an improved benefit/risk ratio compared to tPA, due to potential improved efficacy (based on direct-acting mechanism) and improved safety (based on rapid alpha 2-antiplasmin inactivation, decreased bleeding risk, and neurovascular protective effect).

POTENTIAL ADVANTAGES Microplasmin is a direct-acting thrombolytic that dissolves blood clots without the need for free plasminogen in the circulation. Conversely, all currently approved thrombolytics are indirect-acting plasminogen activators (PAs), requiring conversion of endogenous plasminogen into plasmin in order to achieve thrombolysis. This difference in mode of action may be particularly critical in older clots, in which plasminogen has been partially or fully depleted. Its direct mode of action therefore may enable microplasmin to dissolve clots more predictably, efficiently and quickly than indirect thrombolytics. Microplasmin may also possess improved safety characteristics compared to PAs. Once microplasmin leaves the site of the clot and enters the systemic circulation, it is rapidly inactivated by alpha 2-antiplasmin. Therefore, the potential risk of bleeding may be minimized compared to plasminogen activators, which are not inactivated rapidly in the systemic circulation. An additional potential advantage in treatment of acute ischemic stroke is the lack of neurotoxicity, whereas tPA has been demonstrated to have neurotoxic properties.

PRECLINICAL STUDIES Pharmacology experiments have been performed in both local clot dissolution (canine circumflex artery copper coil–induced thrombosis model and rabbit extracorporeal loop model), as well as numerous acute stroke models. The local clot dissolution models have shown clear thrombolytic efficacy. In the acute stroke models, various insults (thrombotic and mechanical obstruction) were evaluated, as well as in various species (mouse, rat, rabbit). These studies generally demonstrated infarct size reduction and/or improvement of neurological dysfunction, with statistically significant benefit observed out to 10 hour window, without increase in bleeding tendencies. Less associated hemorrhage risk than equieffective doses of tPA has also been demonstrated. In addition, in a rat suture model, unlike tPA, microplasmin demonstrated a vascular protective effect (based on assessment of vascular basement membrane integrity and haemoglobin extravasation). The data from these and other studies provide the rational for the use of microplasmin for treatment of acute vascular occlusions, including acute ischemic stroke, acute peripheral arterial occlusive disease (PAOD) and deep vein thrombosis (DVT).

CLINICAL STUDIES In a Phase I study, microplasmin IV administration was well tolerated at doses up to 4 mg/kg. Phase IIa trials are ongoing in numerous settings, including acute ischemic stroke (MITI–IV and MITI–IA trials) and acute PAOD (MITA trial).

Keywords: thrombolysis, arterial occlusion, stroke
Protein C/Plasminogen and Infection

**ID:** 6

**Thrombosis and thrombolysis in host defense from microbial pathogens**

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Group A streptococci (GAS), a common human pathogen, secrete streptokinase, which activates the host’s fibrinolytic protease, plasminogen. Streptokinase is highly specific for human plasminogen, exhibiting little or no activity against other mammalian species, including mouse. We have generated mice expressing human plasminogen and shown that the mouse protein can largely substitute for the function of its murine ortholog. However, mice expressing human plasminogen exhibit markedly increased mortality following infection with human pathogenic GAS, and this susceptibility is dependent on bacterial streptokinase expression. Thus, streptokinase is a key pathogenicity factor and the primary determinant of host species specificity for GAS infection. In addition, these findings implicate local fibrin clot formation in host defense against a variety of microbial pathogens. Such interactions between host and pathogen may be a key selective pressure leading to a high degree of variability in hemostatic factor function across human populations and contributing to genetic susceptibility to thrombosis.

**Keywords:** Streptokinase, microbial pathogenesis, plasminogen
Protein C

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Discovery of human Protein C genetic severe deficiency indicated that this plasma protein is a physiologically essential antithrombotic, anti-inflammatory factor. Activated protein C (APC), a serine protease, was characterized as a potent anticoagulant and evaluated for its therapeutic properties. In the phase III PROWESS trial, recombinant APC reduced mortality due to severe sepsis, leading to its FDA-approval for treating severe sepsis. In preclinical animal studies, APC prevents neutrophil activation in LPS-induced lung injury, improves islet transplantation outcome in a diabetic mouse model, promotes angiogenesis and wound healing, and provides neuroprotection in rodent stroke models. The molecular mechanisms for various in vitro and in vivo effects of APC involve two APC receptors, protease activated receptor–1 (PAR–1) and endothelial protein C receptor (EPCR). Independent of anticoagulant action, APC acts directly on cells and alters gene expression profiles, inhibits apoptosis, and down-regulates inflammation. As presented in the symposium on stroke studies at this meeting by Berislav Zlokovic, the neuroprotective actions are, at least in part, independent of APC’s anticoagulant activity. Rodent stroke studies and other data highlight the potential direct actions of APC on brain endothelial cells and neurons via PAR–1 and EPCR. Remarkably, APC reduces the neurotoxicity of tissue plasminogen activator (tPA). Thus, APC is a promising therapeutic agent for ischemic stroke. In the PROWESS trial, APC therapy conveyed an increased risk of serious bleeding. Therefore, we sought to reduce bleeding potential but retain APC’s favorable cellular interactions via molecular engineering. Functional proteomics analysis permitted dissection of APC’s anticoagulant activity from its cytoprotective activity based on the specificity of APC’s exosites for its various substrates, factor Va or PAR–1, and cofactors, protein S or EPCR. Two recombinant APC variants were engineered to exhibit only 4–15% anticoagulant activity but normal cytoprotective activity. Alternatively, APC variants were also engineered to manifest normal or enhanced anticoagulant activity but markedly reduced direct effects on cells. These various APC mutants may permit us to clarify whether the anticoagulant activity or the direct cellular effects of APC plays a dominant role and is responsible for the various beneficial biologic effects of APC in various injury model systems. Moreover, current studies address the potential therapeutic use of certain APC variants with reduced anticoagulant activity that may reduce serious bleeding risks while providing the beneficial effects of APC acting directly on cells.

**Keywords:** protein C, thrombosis, apoptosis
Protein C at the Crossroads of Coagulation and Inflammation

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It is well accepted that coagulation and inflammation are intimately related. Both processes are activated in response to an inflammatory challenge. Inflammation initiates coagulation through upregulation of tissue factor expression and downregulation of the fibrinolytic and the Protein C (PC) anticoagulant pathways, leading to procoagulant phenotype. The anticoagulant, activated PC (aPC) is one of the natural occurring inhibitors of blood coagulation. It also possesses antithrombotic, profibrinolytic, antiinflammatory, and antiapoptotic properties. Recently, it has been shown that the level of this protein is an important marker of host sepsis/endotoxemia responses. Unfortunately, homozygous PC deficient (PC−/−) mice die shortly after birth, thus precluding the use of mature mice of this genotype for experiments aimed at assessing the role of PC in disease in vivo. In view of this, we recently generated lines of mice that are genetically predisposed to very low levels of PC expression. These mice showed spontaneous phenotypes related to thrombosis and inflammation. The onset and severity of these phenotypes vary significantly, and are strongly dependent on plasma PC levels. Studies with these mice provide the first direct evidence that maternal PC is vital for sustaining pregnancy beyond 7.5 dpc by regulating the balance of coagulation and inflammation during trophoblast invasion. In addition to their spontaneous phenotypes, very low expressing PC mice developed severe thrombosis and showed persistent inflammation following a bolus injection of low dose LPS. This studies demonstrate that very low endogenous levels of PC predispose mice to early onset disseminated intravascular thrombosis (DIC), thrombocytopenia, hypotension, organ damage, and reduced survival. Furthermore, reconstitution of recombinant aPC enhances survival likely by preventing hypotension induced–organ damage. These findings collectively support the role of aPC as a modulator of coagulation and inflammation.

Keywords: Protein C, Inflammation, Coagulation
Membrane Proteases

Membrane serine proteases and cell morphogenesis

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Limited proteolysis by serine proteases is a critical component of complex biological systems, such as coagulation, fibrinolysis, complement activation and digestion. The S1 tryptic serine proteases are characterized by a triad of histidine (His), aspartate (Asp) and serine (Ser) residues in the catalytic domain, which are necessary for proteolytic activity. A subfamily of these serine proteases has attracted recent interest because they are tethered to the plasma membrane of a variety of cell types, rather than directly secreted into the extracellular space. These membrane-anchored serine proteases have been implicated in diverse cellular functions, including apoptosis, cell differentiation and tumor growth. Some of these, specifically Testisin, prostatin, ?-tryptase and pancreasin, possess carboxy-terminal hydrophobic extensions serving as their membrane anchors. The biology of this subgroup of membrane serine proteases is only beginning to be elucidated. For example, Testisin is a tryptic serine protease anchored at the plasma membrane via a glycosylphosphatidylinositol (GPI)–linker and is expressed on the surface of capillary endothelial cells, eosinophils, and male germ cells. Like other GPI anchored proteins, Testisin may be compartmentalized within cholesterol-rich lipid rafts. Over-expression of Testisin is associated with advanced stage disease in ovarian carcinomas and absence of Testisin expression is associated with testicular tumor formation and/or progression. Targeted deletion of Testisin in mice reveals a critical role for Testisin in processes associated with cell morphogenesis. Testisin KO sperm show defects in sperm morphogenesis evidenced by aberrant sperm cell volume regulation, reduced sperm motility, angulated shape and coiled sperm tails, and increased susceptibility of sperm to decapitation. In addition to its role in sperm morphogenesis, Testisin participates in endothelial cell morphogenesis as occurs during capillary formation in response to angiogenic stimuli. Testisin KO mice show defective angiogenic capillary formation, retarded growth of tumor implants, and aberrant luteal angiogenesis during ovulation. The association of Testisin with specific vascular beds implicates Testisin as a key participant in angiogenesis, highlighting the diversity in function of this membrane serine protease subgroup.
The pro-uPA activator matriptase: key regulator of epidermal development and epithelial carcinogenesis

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Matriptase (MT-SP1, epithin, ST14, and TADG-15) is an epithelial–specific transmembrane serine protease frequently overexpressed in human cancers. We have determined the expression, physiological functions, and contribution of matriptase to carcinogenesis by matriptase gene targeting, enzymatic gene trapping, and matriptase transgenesis in mice. Matriptase gene deletion unveiled an essential role of the membrane serine protease in oral epithelial and epidermal differentiation, hair follicle growth, and thymic development. Matriptase–deficient mice died shortly after birth due to incomplete formation of the stratum corneum lipid matrix and the cornified envelope of corneocytes, which lead to loss of epidermal barrier function and fatal dehydration. These defects of matriptase–deficient epidermis were linked at the molecular level to the complete abrogation of the proteolytic processing of the large epidermal polyprotein profilaggrin into filaggrin monomer units and the N-terminal filaggrin S–100 regulatory protein, which translocates to the nucleus to promote terminal epidermal differentiation. Matriptase localization studies using enzymatic gene trapping revealed a close association between the expression and function of the membrane serine protease. Thus, in the epidermis, oral epithelium, and thymic epithelium, matriptase was exclusively expressed in post–mitotic transitional layer cells in the process of undergoing terminal differentiation. In all three tissues, matriptase colocalized with profilaggrin, frequently displaying close proximity to profilaggrin–containing granules, as revealed by ultrastructural analysis. Likewise, in accordance with the important function of matriptase in hair follicle growth, matriptase was specifically expressed in growth (anagen) phase hair follicles and was located in a population of undifferentiated and rapidly proliferating hair matrix cells. Enzymatic gene trapping revealed that matriptase was expressed at comparable levels in all stages of malignant progression during chemically induced multistage carcinogenesis. However, the membrane protease displayed a dramatic spatial redistribution during the transition of epidermal lesions from hyperplasia to dysplasia. Thus, matriptase expression was narrowly confined to highly differentiated, non–proliferating keratinocytes in normal and hyperplastic epidermis. In contrast, dysplastic and malignant lesions presented expression of matriptase in a much broader subset of keratinocytes, including a population of proliferating, keratin-5–positive basal keratinocytes with high self–renewal capacity that include epidermal stem cells believed to be the primary target cells for squamous cell carcinogenesis. This spatial dysregulation of matriptase indeed may be functionally relevant to epithelial carcinogenesis, as transgenic expression of matriptase, even at modest levels, in these keratin-5 positive keratinocytes sufficed to cause both spontaneous epidermal carcinomas and to dramatically potentiate the effect of carcinogen exposure. Taken together, these studies have revealed a key role of the type II transmembrane serine protease matriptase in epidermal development and carcinogenesis.

Keywords: matriptase, cancer, epidermal development
The semaphorins are a family of proteins originally identified as regulators of axon growth that have recently been implicated in cell migration, immune responses and tumor progression. The plexins are high-affinity receptors for the semaphorins and are responsible for initiation of signaling upon ligation. For example, Plexin-B1, the receptor for Semaphorin 4D, influences the activation of the Rac and Rho GTPases to induce changes in actin cytoskeletal organization. In endothelial cells, Semaphorin 4D–mediated Plexin-B1 signaling initiates chemotaxis, a mechanism likely employed by transformed cells to induce angiogenesis. However, in order to exert pro-angiogenic functions in vivo, Semaphorin 4D, a cell surface protein, must be processed and released from tumor and/or stromal cells and act in a paracrine manner on endothelial cells. Through the use of specific protease inhibitors and knockout MEFs, we show that membrane type 1–MMP, a member of a family of metalloproteinases that has been linked to the processing of certain membrane–anchored cytokines, cytokine receptors and adhesion molecules, is necessary for the proteolytic processing of Semaphorin 4D into its soluble form. We found that MT1–MMP, which is not expressed in normal or immortal but non–tumorigenic epithelial cell lines, was present in several head and neck squamous cell carcinoma cell lines. Through the use of shRNA technology, we determined that MT1–MMP was necessary for Semaphorin 4D shedding by these cells and induction of blood vessel growth in vivo, suggesting that the pro–angiogenic effects of Semaphorin 4D by tumors are controlled by MT1–MMP.

Keywords: MT1-MMP, semaphorins, angiogenesis
Neurosciences

ID: 218

Tissue plasminogen activator and the interplay between neurons and microglia

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Tissue plasminogen activator (tPA) is a serine protease expressed in the mouse brain by neurons and immunocompetent microglia. Mice deficient in tPA exhibit resistance to excitotoxic neuronal injury and impaired microglial activation. tPA acts to activate microglia by interacting with annexinA2 on the surface of these cells. Activated microglia migrate to the sites of neuronal injury by following transient gradients of chemokines, one of which (that formed by monocyte chemoattractant protein–1, MCP–1, CCL2) is modulated by the presence of an active tPA/ plasmin proteolytic system. The tPA–mediated activation of microglia is followed by the upregulation of the inducible nitric oxide synthase (iNOS) that generates NO, which in the presence of an oxidative environment, is rapidly converted to peroxynitrite and modifies by nitrosylation proteins on neighboring neurons. Using molecular, pharmacological and genetic tools we investigate the involvement of tPA in different processes of the communication between neurons and microglia in physiology and pathology.

Keywords: microglia, blood brain barrier, proteolysis
**Tissue Plasminogen Activator in Nervous System Function and Dysfunction**

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In the early 1990s, it became clear that tissue plasminogen activator (tPA) is expressed in the mammalian brain, and can participate in the function and dysfunction of the nervous system. I will discuss the role of tPA in various neuronal pathologies, including neuronal degeneration in the central nervous system, demyelination in the peripheral nervous system, stress, and Alzheimer’s disease.

**Keywords:** tPA, stress, neurons
Control of Vascular Wall Integrity by LRP1

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The low-density-lipoprotein receptor–related protein 1 (LRP1) is a member of the LDL receptor gene family. This evolutionarily highly conserved class of cell surface lipoprotein receptors has numerous and diverse roles in metabolism, atherosclerosis, development and intercellular communication. However, lipoprotein and cholesterol transport is only one function of this evolutionarily ancient receptor family. We now realize that the regulation of a wide range of fundamental cellular signaling pathways plays a major role in the biological activity of this gene family. We have shown a pivotal role for LRP1 in the control of the platelet–derived growth factor receptor beta (PDGFR≤) signaling in the vascular wall. Somatic deletion of LRP1 in vascular smooth muscle cells results in overactivation of PDGFR≤ activity, increased mitogenic response, thickening of the vascular wall and disruption of the elastic fiber layers. As a result of this hyperactive proliferative response and increased vascular remodeling, mice lacking LRP1 in their smooth muscle cells are extremely sensitive to cholesterol–induced atherosclerosis. We have used a range of experimental, including pharmacologic and genetic strategies to analyze how PDGFR≤ and LRP1 control atherosclerotic lesion progression in the vascular wall in the mouse on the local, somatic level. These experiments have revealed novel mechanisms by which a lipoprotein receptor functions as an atheroprotective signal integrator to control vascular repair and remodeling directly in the vessel wall.
The role of the urokinase/urokinase receptor system in pulmonary immune and inflammatory responses.

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Neutrophils and lymphocytes express urokinase–type plasminogen activator (uPA) and the urokinase receptor (uPAR, CD87). uPA and uPAR have been implicated in the in vitro modulation of cellular recruitment, and in some activation processes. Our work has focused on the role that uPAR and uPA play in the modulation of pulmonary immune and inflammatory responses, and host defense. In vitro, uPAR modulates beta-2 integrin function. Pseudomonas aeruginosa infection recruits neutrophils to the lungs by a beta-2 integrin–dependent mechanism. We demonstrate that mice deficient in uPAR (uPAR−/−) have profoundly diminished neutrophil recruitment in response to P. aeruginosa pneumonia, compared to Wild Type (WT) mice. The requirement for uPAR is independent of uPA, as recruitment in uPA−/− mice is indistinguishable from recruitment in WT mice. However, bacterial killing is diminished in absence of uPA. The generation of superoxide and degranulation responses are profoundly reduced in uPA−/− neutrophils. Thus, the uPA/uPAR system modulates several of the crucial steps in neutrophil activation that result in bacterial killing and effective innate host defense.

Activated lymphocytes and monocyte/macrophages express uPA and uPAR. uPA is hypothesized to provide proteolytic activity enabling immune cells to traverse tissues during recruitment, and has been implicated as a cytokine modulator in vitro. We evaluated the importance of uPA in protective immune responses to C. neoformans. C. neoformans was inoculated into the lungs of uPA−/− and WT mice and cell recruitment to the lungs quantitated. Colony-forming units (CFU) in lung, spleen and brain were determined to assess clearance, and survival curves determined. By day 21 post inoculation, uPA−/− mice had markedly fewer pulmonary CD4+ T lymphocytes and macrophages compared to WT; pulmonary CFUs in the uPA−/− mice continued to increase, whereas CFUs diminished in WT mice. In survival studies, only 3/19 WT mice died whereas 15/19 uPA−/− mice died. Thus, lack of uPA results in inadequate specific immune responses, uncontrolled infection, and death. Since effective host defense against C. neoformans requires the generation of a T1 immune response, we determined the role of uPA in these processes. T lymphocyte responses and proliferation were quantified, and cytokine concentrations determined by ELISA. Regional lymph nodes and lungs of infected uPA−/− mice contained fewer CD4+ cells than WT mice, despite normal lymphocyte recruitment, suggesting impaired T cell proliferation in response to the pathogen in vivo. In vitro, uPA−/− T lymphocytes had impaired proliferative responses compared to WT. Infected WT mice generated T1 cytokines in the lung, characterized by high levels of IFN-gamma and IL-12. uPA−/− mice had markedly decreased levels of IFN-gamma and IL-12. In the absence of uPA, the cytokine profile of regional lymph nodes shifted from a T1 pattern characterized by IFN-gamma and IL-2, to a weak, nonpolarized response. Thus, in the absence of uPA, lymphocyte proliferative responses are diminished, and mice fail to generate protective T1 cytokines, resulting in impaired antimicrobial activity. Our work has shown that uPA is a critical modulator of immune responses and of immune cell effector functions in vivo.

Keywords: inflammation, immune responses, urokinase
Role of Tissue Factor, Coagulation Proteases and PARs in Inflammation

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A blood clot is the end–product of a proteolytic cascade containing several serine proteases, such as Factor VIIa, Factor Xa and thrombin. This cascade is initiated by the binding of Factor VII/VIIa to the cellular receptor tissue factor (TF). Recently, it was shown that coagulation proteases cleave and activate a family of cellular receptors, called protease activated receptors (PARs). There are four members of this family, PAR1–4. Thrombin activates PAR1, PAR3 and PAR4; Factor Xa activates PAR1 and PAR2; and Factor VIIa activates PAR2. Importantly, thrombin activation of platelets is mediated by PARs. In addition, cleavage of PAR1 and PAR2 on endothelial cells by coagulation proteases activates various intracellular signaling pathways and induces the expression of inflammatory mediators. These results suggested that PARs may mediate the crosstalk between the coagulation protease cascade and inflammation. The roles of tissue factor, coagulation proteases, and PAR1 and PAR2 in coagulation and inflammation in different animal models will be discussed.
Multifunctional roles of plasmin in inflammation: matrix degradation and much more

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The Plasminogen activator (PA) system and plasmin have been suggested to be involved in the degradation of extracellular matrix (ECM) and tissue-remodeling during a number of autoimmune and non-autoimmune inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), wound healing and infection. Traditionally, the phenotypes of the plasminogen (plg)-deficient mice during various disease models have mainly been attributed to defect in cell migration, ECM remodeling or to an inability to proteolytically degrade fibrin. However, many results obtained from studies of plg-deficient mice cannot be adequately explained by these mechanisms. Our studies in animal models of RA and MS have suggested that plasmin may only be essential in autoimmune diseases that are critically dependent on complement activation. The phenotypes of plg-deficient mice are also similar to the phenotypes of some complement factor-deficient mice during autoimmune RA and MS. Such similarities have also been observed in different non-autoimmune disease models. Our results as well as studies by others have shown that extensive necrosis occurs in plg-deficient mice in wound healing and tissue and organ regeneration models. The complement system has been shown to be critical for clearance of necrosis tissue and mice carrying complement factor deficiencies have similar phenotypes in these models, suggesting a possible coupling between plasmin and the complement system. The functional roles of plg in the host defense against bacterial infections include a role in inflammatory cell migration as well as interactions of host plg with PAs expressed by pathogens. In our studies of bacterial-induced arthritis, we find that the number of bacteria that accumulate in the joints of plg-deficient mice are much higher than in wild-type controls. Both IL-6 and IL-10 levels are also significantly lower in plg-deficient mice as compared to wild-type control mice. These findings are in line with the phenotypes of some complement factor-deficient mice, but can not be easily explained by previously proposed roles of plasmin. Taken together, our results suggest that plasmin play multifunctional roles in inflammation.
Angiogenesis

**ID:** 58*

**The C-terminal globular domain of fibrinogen gamma chain suppresses angiogenesis and tumor growth**

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Fibrinogen is a major plasma protein (350 Kd) that induces proliferative signals by serving as a scaffold to support the binding of growth factors and to promote the cellular responses of adhesion, proliferation, and migration during wound healing, angiogenesis, and tumor growth. Plasma concentrations of fibrinogen degradation products (FDPs) generated during fibrinolysis are markedly elevated in the adult respiratory distress syndrome, disseminated intravascular coagulation, and septic shock, and FDPs are implicated in tissue injury associated with these disorders. However, the basis of how FDPs induce the vascular injury is unclear. The fibrinogen gamma chain has a C-terminal globular domain (gammaC, residues 151–411 of gamma chain, 30 Kd) to which several integrin cell adhesion receptors (e.g., platelet alphabbbeta3, endothelial alpahbeta3, and leukocyte alphambeta2) bind. Integrins play a critical role in signal transduction from fibrinogen. We found that gammaC and its truncation mutant (designated gammaC399tr), with a deletion of the C-terminal 12 residues, effectively blocked proliferation of endothelial cells in tissue culture at concentrations of less than 10 micro g/ml, while native fibrinogen and fragment D did not have a noticeable effect. We demonstrated that the effect of gammaC is due to induction of apoptosis, as evidenced by the detection of apoptotic cells by using fluorescence-labeled annexin V and of activated caspase 3/7 in gammaC-treated cells. GammaC also blocked tube formation from endothelial cells in matrigel. GammaC did not induce such effects in several other cell types tested (e.g., CHO cells, keratinocytes, and tumor cells). We demonstrated that tumor cells that secrete gammaC or gammaC399tr grew much slower than non-secreting control cells in vivo. Also recombinant gammaC399tr protein markedly suppressed tumor growth, development of intratumoral vasculature, and tumor metastasis when injected intraperitoneally in vivo. We showed that the structural determinant responsible for binding to endothelial cells is cryptic in native fibrinogen, but is exposed in gammaC. We showed that the alpahbeta3-binding site is also cryptic in fibrinogen and Fragment D, but is exposed in gammaC. However, we were unable to determine whether integrins are directly involved in gammaC–induced apoptosis of endothelial cells, since antagonists and antibodies to integrins induce apoptosis of endothelial cells. In conclusion, these results suggest that fibrinogen has a novel cryptic determinant that can exert apoptosis–inducing activity on endothelial cells when exposed, and polypeptides containing this determinant have therapeutic potential. 1. Fitzgerald, D.J. Fibrinogen receptor and platelet signalling. Blood Coagul Fibrinolysis 10 Suppl 1, S77–9 (1999), 2. Drews, R.E. Critical issues in hematology: anemia, thrombocytopenia, coagulopathy, and blood product transfusions in critically ill patients. Clin Chest Med 24, 607–22 (2003). 3. Marder, V., Martin, S., Francis, C. & Colman, R. Consumptive thrombohemorrhagic disorders. In Hemostasis and Thrombosis. (eds. Colman, R., Marder, V., Salzman, E. & Hirsh, J.) 975–1015 (J. B. Lippincott Company, Philadelphia, 1987). 4. Haynes, J.B., Hyers, T.M., Giclas, P.C., Franks, JJ. & Petty, T.L. Elevated fibrinogen degradation products in the adult respiratory distress syndrome. Am Rev Respir Dis 122, 841–7 (1980).

**Keywords:** fibrinogen, apoptosis, endothelial cells
Adenovirus Mediated Expression of Tissue Factor Pathway Inhibitor-2 Inhibits Endothelial Cell Migration and Angiogenesis

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Aberrant angiogenesis plays a pivotal role in tumor growth and metastasis, cardiovascular diseases, chronic inflammation and diabetes. Extracellular matrix (ECM) remodeling during angiogenesis is largely accomplished through plasmin-dependent pericellular proteolysis and matrix metalloproteinases (MMPs). Tissue factor pathway inhibitor–2 (TFPI–2) is a Kunitz–type protease inhibitor with prominent ECM localization, which inhibits plasmin and reduces MMP activity. Here we investigated the role of TFPI–2 in endothelial cell (EC) migration, proliferation, ECM turnover and angiogenesis. First we determined that endogenous TFPI–2 expression (mRNA and protein) was up–regulated in migrating EC. We next investigated the effect of TFPI–2 overexpression via replication–deficient adenovirus (AdCMVTFPI–2) infection of the EC line EA.hy926 on the proliferation, migration and capillary tube formation in Matrigel. Cell migration, ECM turnover and angiogenesis were also studied in vivo using Matrigel or polyvinyl alcohol sponge implant models. EC proliferation and lateral migration in vitro were impaired by AdCMVTFPI–2 and by exogenous TFPI–2 protein. Zymography of migrating cells revealed TFPI–2–dependent plasmin inhibition. Capillary–like structure formation was prevented by TFPI–2. In vivo, AdCMVTFPI–2 inhibited cell proliferation and reduced VEGF–dependent neovascularization. Enhanced ECM and fibrin deposition were detected in the sponge implants of AdCMVTFPI–2 treated mice. Increased apoptosis occurred in both AdCMVTFPI–2–treated EC in vitro and in the in vivo implants. Our data demonstrate that adenovirus–mediated overexpression of TFPI2 inhibits cell migration and angiogenesis and enhances apoptosis and extracellular matrix deposition. These data suggest the potential clinical utility of TFPI–2 to inhibit cell invasiveness and pathological angiogenesis.

Keywords: TFPI2, Endothelial cells, Angiogenesis
Novel peptide scaffold promotes cell migration and angiogenesis through enhanced expression of angiogenic factors

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Introduction. Tissue engineering is a rapidly developing area of research with potential to provide alternative tissues and treatments for a variety of clinical conditions. Ability to manipulate and promote angiogenesis is crucial for most of tissue engineering applications. Depending on the substrate, presence of support cells, such as fibroblasts, and exogenous angiogenic factors, angiogenesis in engineered tissues and in vitro models can be either promoted or inhibited by the matrix proteolysis and substrate degradation. Recently, we have reported that a novel cell-native biomaterial – self-assembling peptide scaffold – supports capillary-like network formation, promotes angiogenesis and inhibits endothelial cell apoptosis in the absence of exogenous angiogenic factors. The goal of the present study was to test the hypothesis that self-assembling peptide scaffold promotes angiogenesis and cell migration through enhanced expression of angiogenic factors angiopoitin 1 (Ang1), vascular endothelial growth factor (VEGF) and proteolytic factors matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) in three-dimensional cultures of endothelial cells and fibroblasts.

Methods. Human microvascular endothelial cells and human dermal fibroblasts (Cascade Biologics) were cultured on gelatin-coated dishes. Cells (passage 4–9) were trypsinized, and embedded in 1% RADi-16 peptide scaffold either as mono-cultures or as mixed co-cultures (1:1 ratio of endothelial cells and fibroblasts) and cultured for up to 7 days. Cells embedded in the collagen type I gel served as controls. Medium samples were collected on days 1–6, and concentrations of Ang1, VEGF, MMP-2 and MMP-9 were determined as pg/ml using ELISA assays (R&D Systems). At day 7, samples were embedded in paraffin. Serial 5 um-thick sections were used to quantify cell apoptosis (TUNEL assay, Roche).

Results & Discussion. In both monocultures and co-cultures with fibroblasts, endothelial cells migrated and formed single cell lumens and multi-cell capillary-like structures by day 3. In contrast, fibroblasts did not form lumens, but spread out. The peptide scaffold had a protective effect against apoptosis of endothelial cells, with much lower levels of apoptosis detected up to day 7 of culture, as compared with collagen type I gel controls (p<0.01, ANOVA). These results demonstrate that endothelial cells embedded in the peptide scaffold with or without fibroblasts undergo activation and change phenotype in a manner similar to the process of sprouting in vivo. ELISA results showed significantly increased expression of Ang1 and VEGF in all peptide scaffold cultures, relative to the collagen type I cultures (p<0.01, ANOVA, n=4). MMP-2 expression in the peptide scaffold cultures containing fibroblasts was higher, compared with endothelial cells only and collagen type I cultures. In contrast, MMP-9 expression was increased for all peptide scaffold cultures only at days 3&4 and was similar to collagen cultures for other time points. These results indicate that increased expression of VEGF and Ang1 in the peptide scaffold may be the mechanism responsible for spontaneous angiogenesis and improved cell survival, in contrast to collagen type I gels. Our results also indicate that MMP-2 is likely involved in fibroblast, but not endothelial cell, migration through the peptide scaffold, while MMP-9 may be important for migration of both cell types.

Keywords: peptide scaffold, angiogenic factor, MMP

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Disease processes may be reflected by correlations among tissue kallikrein proteases but not with proteolytic factors uPA and PAI-1 in primary ovarian carcinoma

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Purpose. In epithelial ovarian cancer, the high mortality rate is usually ascribed to late diagnosis since epithelial ovarian tumors commonly lack early warning symptoms. Tumor-associated biomarkers, useful for diagnosis, prognosis, and/or therapy response prediction are in short supply. The serine protease urokinase-type plasminogen activator and its inhibitor PAI-1, both members of the plasminogen activation system of fibrinolysis, and the recently described members of the interfacing system of tissue kallikrein serine proteases (hK1 to hK15) were reported to be associated with malignancy and tumor progression of early and/or advanced stage ovarian cancer patients. Materials and methods. We determined simultaneously by ELISA the protein content of 9 proteolytic factors (uPA, PAI-1, and tissue kallikreins hK5, 6, 7, 8, 10, 11, 13) in detergent-released extracts of 142 ovarian cancer patient primary tumor tissue specimens and conducted a direct comparison of protein expression levels of these tumor tissue-associated factors which has not been performed so far. The numbers obtained were weighted statistically and analyzed according to protein expression levels, FIGO stage, and nuclear grading.

Results. There was no difference in expression levels of any of the factors examined regarding to FIGO stage, except for hK5 which was expressed at a higher level in tumor tissues of FIGO III/IV rather than in FIGO I/II patients. hK5 was also more expressed in undifferentiated G3 tumors than in G1/2 classified ovarian cancer patient tumor specimens with higher level of cellular differentiation. We observed that hK5, 6, 7, 8, 10, and 11 were correlated with each other at a statistically significant level and that the highest level of statistically significant correlation was attributed to low-grade (G1) ovarian cancer patients. On the other hand, uPA, PAI-1, and hK13, respectively, were correlated to a few of the other proteolytic factors only, and if so, at a low level of association.

Conclusion. These data further support the notion that several of the tandemly colocalized genes of the tissue kallikrein family on locus 19q13.4 are co-expressed in ovarian cancer patients, substantiating the possible existence of a steroid hormone-driven tissue kallikrein cascade in patients with malignant disease.

Keywords: tissue kallikreins, uPA/PAI-1, ovarian cancer
Effects of overexpression of the human tissue kallikrein genes KLK4, 5, 6, and 7 on the malignant phenotype of ovarian cancer cells

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The human tissue kallikrein family encompasses 15 serine proteases. Most of the tissue kallikrein genes (KLK1–15) or proteins (hK1–15) have been found to be either under- or overexpressed in certain carcinomas, especially in breast, prostate, testicular and ovarian cancer. In ovarian cancer tissue, several tissue kallikreins are strongly over- or even de novo expressed, compared to normal ovarian tissue. This may indicate that these tissue kallikreins participate in an enzymatic cascade pathway which is strongly activated in ovarian cancer. In the present study, full length cDNAs encoding hK4, hK5, hK6, and hK7 were amplified from ovarian cancer tissue and cloned into the eukaryotic expression vector pRcRSV. OV–MZ–6 ovarian cancer cells, which do not express any of the four tissue kallikreins, were stably co–transfected with these plasmids resulting in the tumor cell line OV–KLK4+5+6+7. Applying sensitive ELISA formats for hK4, hK5, hK6 and hK7, we demonstrated that the stably transfected OV–KLK4+5+6+7 cell line secreted detectable amounts of the respective tissue kallikreins into the medium, ranging from about 0.5 to 20 ng/ml after growth in culture for 48 h. OV–KLK4+5+6+7 cells displayed similar proliferative capacity as the vector–transfected control cells (OV–RSV), but showed a significantly increased invasive behavior in the in vitro Matrigel invasion assay (p<0.01; Mann–Whitney U–test). For in vivo analysis, cells expressing the four tissue kallikreins were inoculated into the peritoneum of nude mice. Simultaneous expression of hK4, hK5, hK6, and hK7 resulted in a remarkable 92% mean increase of tumor burden compared to the vector–control cell line. Five out of 14 mice in the “tissue kallikrein overexpressing” group displayed a tumor/situs ratio of greater than 0.198, while this weight limit was not at all exceeded in the vector control group consisting of 13 mice (p=0.017; chi–square–test). Our results strongly support the view that tumor–associated overexpression of tissue kallikreins contributes to ovarian cancer progression.

Keywords: tissue kallikreins, ovarian cancer, serine protease
Fibroblast Activation Protein Confers Enhanced Motility in a Fibroblast Derived 3D Matrix System


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**Purpose:** Fibroblast activation protein (FAP), also known as seprase and anti-plasmin converting enzyme, is a 97 kDa type II integral membrane glycoprotein belonging to the serine protease family. Human FAP is unique in its selective expression by tumor stromal fibroblasts in epithelial carcinomas, but not commonly seen on epithelial carcinoma cells, normal fibroblasts, or other normal cells. FAP has in vitro dipeptidyl peptidase and endopeptidase activities. We have previously shown in an epithelial xenograft model that FAP overexpression potentiates tumor growth, and abrogation of its enzymatic activity attenuates FAP-driven tumor growth. In addition we have described that FAP overexpression enhances motility in a 2-dimensional wound healing assay. We wished to extend these observations seen in FAP-expressing epithelial cells by studying the effects of FAP expression on both fibroblasts and their extracellular matrices (ECM) in an in vivo–like 3-dimensional (3D) matrix system.

**Methods:** NIH–3T3 fibroblasts transfected with an rtTA tetracycline induced promoter to conditionally express FAP (NIH3T3–FAP) and NIH3T3–Vector fibroblasts were used to derive 3D matrices. Fibroblasts were plated to confluency for 8 days, and the fibroblast–derived matrices were then denuded of cells with alkaline detergent treatment (extracted matrices). Time–lapse motility assays were performed using NIH3T3–FAP or NIH3T3–Vector cells re–plated within 3D–matrices and analyzed. The overall motility rate of cells were calculated by tracking their movements over time (T, for trajectory), and compared to net distance (D). The calculation of D/T indicated the directionality of the individual cell movement, and the community mean for movement over the course of the experiment was determined using Metamorph 6.3 software.

**Results:** Morphologically, unextracted 3D cultures of NIH3T3–FAP cells showed increased spindle–shape and cell organization that are characteristic of tumor associated stroma, compared to NIH3T3–Vector cultures. Overexpression of FAP on fibroblasts resulted in a 1.6–fold increase in velocity and greater directionality compared to vector alone (D/T .59 vs .32, respectively). Matrices made from FAP expressing fibroblasts induced a 1.4–fold increase in velocity compared to NIH–Vector derived matrices. NIH–Vector fibroblasts exposed to NIH3T3–FAP conditioned media had enhanced path distance and directionality similar to FAP–expressing fibroblasts, suggesting that FAP–expressing fibroblasts might confer paracrine effects enhancing cell directionality.

**Conclusion:** We describe a 3D system that morphologically recapitulates many of the features of tumor stroma in vivo. We demonstrate that FAP expression in fibroblasts results in enhanced directional motility within 3D matrices. We hypothesize that FAP expressing fibroblasts may alter the tumor microenvironment to facilitate directional epithelial tumor invasion. Additional studies are being performed to study the interactions between FAP fibroblasts, the extracellular matrix, and malignant epithelial cells in fibroblast–derived in vivo–like 3D matrices.

**Keywords:** stromal fibroblasts, fibroblast activation protein, tumor motility
Synthesis and the biological activity of the phosphonic inhibitors of urokinase type plasminogen activator

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The urokinase plasminogen activator (urokinase, uPA) is a trypsin–like serine protease, important in many cellular processes such as tumorigenesis, cell proliferation and migration, cell adhesion, angiogenesis, intravasation and metastasis. The primary role of uPA is to convert plasminogen into its active form plasmin, a broad spectrum serine protease which in turn activates matrix metalloproteases, and also degrades several extracellular matrix components, including laminin, collagen type IV, fibrin, and fibronectin. It facilitates the detachment of cancer cells from the primary tumor, and their migration within the surrounding tissue into blood and lymph vessels leading to the formation of metastases at distant sites. Alpha-aminoalkylphosphonate diphenyl esters, the phosphonic analogues of naturally occurring amino acids, and their peptidyl-analogues comprise a group of irreversible, slow binding, competitive inhibitors for chymotrypsin–like and trypsin–like serine proteases such as chymase, trypsin, elastase or urokinase. We have focused our investigations on development of new low molecular weight phosphonic inhibitors for uPA which would provide to new therapeutics. Some of the compounds inhibit uPA at nanomolar concentration with high selectivity toward trypsin. First results of the initial structure activity relationship experiments lead to the most potent phosphonic uPA inhibitors synthesized to date. Using molecular modeling methods structures of some phosphonic inhibitors in complex with the enzyme molecule were optimized. The results from the initial experiments concerning the influence of synthesized inhibitors on the proliferation and migration of cancer cells are also presented as well as the first experiments using the animal models. In conclusion, we will present the synthesis and the biological activity of new Cbz–N–protected derivatives of ω-aminoalkylphosphonates – phosphonic analogues of arginine as potent inhibitors of urokinase and trypsin.

Keywords: urokinase inhibitors, alpha-aminophosphonates, anticancer activity
CLINICAL SIGNIFICANCE AND CO-EXPRESSION OF KLK5 AND KLK7 IN BREAST CANCER

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The family of human tissue kallikreins is a subgroup of serine proteases that has recently been identified. The family consists of 15 members, many of which exhibit an altered expression in various types of cancer, such as prostate, ovarian and breast cancer. Therefore, kallikreins may have a potential role as markers for malignancy. Human kallikrein 5 (KLK5, HSCTE) and 7 (KLK7, HSCCE) are members of the kallikrein family that have been identified from a keratinocyte library. These serine proteases are implicated in the degradation of intercellular cohesive structures in the outermost layer of the skin and contribute to the cell shedding process at the skin surface. It has been proposed that hK5 may act as activator of hK7 in the above process. KLK5 has been shown to be differentially expressed in a variety of endocrine tumors including ovarian, breast and prostate cancer. KLK7 is differentially expressed in ovarian and breast cancer and may be a potent biomarker for these diseases. The purpose of our study was to examine the possible co-expression of the two genes in breast cancer. We developed RT-PCR methods in order to selectively amplify the full length splice variants of KLK5 and KLK7. The mRNA expression of the two genes was examined in a group of 80 breast cancer samples. Actin was used as a house-keeping gene. The PCR products were sequenced in order to verify their identity. The results were correlated to other clinical and histopathological variables as well as to patient outcome. KLK5 was found to be expressed in 30/80 (37.5%) of breast cancer tissues and KLK7 in 26/80 (32.5%). KLK7 was significantly (p=0.04) more frequently expressed in samples that were positive for KLK5 expression. KLK5 expression was more frequent in tumors with low levels of estrogen receptors (p=0.04) and progesterone receptors (p=0.005). KLK7 expression was more frequent in tumors with low levels of estrogen receptors (p=0.04). KLK7 expression was also associated with shorter overall survival. Our results are in agreement with the hypothesis that there is an interplay between KLK5 and KLK7 expression. It is also suggested by the statistical analysis that KLK5 and KLK7 expression is correlated with hormone-independent, and thus, poor prognosis tumors.

Keywords: breast cancer, KLK5, KLK7
Cathepsins in Cancer: Sensitive and Convenient Assays to Measure Their Activities in Biological Samples

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Cathepsins are proteases, which are localized intralysosomally. They play a vital role in many physiological processes and have been implicated in many pathological events including cancer, Alzheimer’s disease, inflammation, rheumatoid arthritis, and multiple sclerosis. Cathepsins are subdivided into serine, cysteine and aspartyl classes on the basis of their active site residue. Similar to other proteases, cathepsins are synthesized as inactive precursors, and are activated by proteolytic removal of N-terminal propeptide. Their activities are regulated by the endogenous inhibitors, intracellular cystatins A and B, and extracellular cystatin C. In cancer, the most studied cathepsins are those of the cysteine and aspartic classes, cathepsin B, L, S, and D. In some cancers these enzymes are often either secreted or associated with plasma membrane. Their activity is increased during tumorigenesis and the inhibition of cathepsin function impairs tumor development. Since cathepsins activity is tightly connected with many diseases there is a need for the precise and specific measurement of their activity in biological samples. We have developed highly sensitive and specific assays to measure cathepsin B, L, S, and D activity in biological samples using small, fluorogenic peptides, AMC-based or FRET substrates. We used Z-RR AMC, Z-FR AMC, and Z-VVR AMC substrates for cathepsin B, L, and S, respectively. Cathepsin D activity was measured with FRET substrate, Mca-GKPIFFRLK(Dnp)-R-NH2. To eliminate the interfering proteolytic activity in biological samples we have included selective enzyme inhibitors, CA 074 and Z-FY(t-Bu)-DMK, or developed a novel activity assay platform, immunocapture activity assay. This novel platform, where an antibody immobilized on 96-well plate is used to capture the enzyme, was used to develop cathepsin D and S assays. All assays are optimized with highly purified native or recombinant cathepsins. Typical detection range of the enzyme varies from 3 to 50 ng/ml. For each assay, in particular for cathepsin B, L, and S, cross-reactivity with other enzyme is minimal. Both type of assays, soluble and immunocapture, are versatile and can be used to measure cathepsin activity in variety of biological samples including cell lysates, tissue extracts, body fluid, and for screening cathepsin inhibitors.

Keywords: cathepsins, activity, assay
Estradiol inhibits EGF-induced mobilization of uPAR from detergent insoluble domains in ERα negative, GPR30 positive ovarian cancer cells.

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EGF stimulates uPAR expression in ovarian cancer cells via three distinct mechanisms: increase of uPAR mRNA, decreased degradation of uPAR, and immediate recruitment of uPAR from detergent resistant domains. Estradiol inhibits EGF induced increase of detergent soluble uPAR. The effects of estrogen in tumor biology are complex and insufficiently understood. Identification of a high affinity membrane bound G-protein coupled estrogen receptor, GPR30, will help to understand certain estrogen effects. Estradiol is not generally considered growth promoting in ovarian cancer, but contradictory effects influencing tumor survival and progression have been reported, and adjuvant treatment with SERM (Selective Estrogen Receptor Modulator) compounds has been tried. Methods: We used seven ovarian cancer cell lines: OVCAR-3, SKOV-3, SKOV-ip, TOV-21, TOV-112, HEY, and ES-2. Cell migration and invasion through uncoated vs. Matrigel coated membranes. ELISA for uPAR. Cellular binding of 125I-uPA, and cellular degradation of 125I-uPA: PAI-1 complex. Real-time PCR, Northern blot, and DNA gel for uPAR and GPR30. Results: EGF stimulated cell migration, invasion, and uPAR expression in all seven ovarian cancer cell lines. These effects of EGF were inhibited by estradiol. We found that this effect of estradiol does neither result from reduced amount of uPAR mRNA, nor from increased degradation of uPAR, or increased shedding of suPAR. Estradiol does, however, reduce mobilization of uPAR from detergent insoluble domains, which is an immediate response to EGF stimulation. These domains are likely to involve lipid rafts. This effect seems to be mediated by the membrane receptor GPR30, since it results from stimulation with not only estradiol but also the “inhibitor” ICI 182780. Furthermore, these cells express GPR30 mRNA but not ER-alpha mRNA or protein. The combined effect of estradiol and ICI 182780 did not exceed the effect of each compound alone. Conclusions: We found that estradiol reduced the response to EGF by inhibiting the immediate mobilization of uPAR from detergent resistant domains, i.e. the lipid rafts, to the cell surface. We suggest that this effect is mediated via GPR30, since these cells are ERα negative GPR30 positive, and the “inhibitor” ICI 182780 had an agonistic effect.

Keywords: Ovarian cancer, GPR30, uPAR
An in vitro model for tumor cell - stromal cell crosstalk in ovarian cancer. Induction of uPA gene expression in stromal cells involves multiple signaling molecules, and is augmented by a 3D extra-cellular matrix.

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We have previously reported that benign and well-differentiated malignant ovarian tumors express uPA mRNA in the epithelial tumor cells. In contrast, poorly differentiated ovarian tumors have massive expression of uPA mRNA in the stroma. This switch to stromal expression of uPA mRNA is associated with loss of histological differentiation. We hypothesize that this is a tumor cell orchestrated phenomenon, which is significant to the phenotype of these tumors. (Borgfeldt, Int J Cancer 79: 588, 1998)

Methods: With the purpose to create a model for ovarian cancer tissue, we have employed ovarian cancer cell lines (SKOV-3, OVCAR-3, ES-2, HEY) in co-cultures with either fibroblastic cell lines (F-434, LEP) or primary endometrial stromal cells (ESC). The cancer cells were in the upper chamber and fibroblastic/stromal (FS) cells in the lower chamber in a two-chamber tissue culture system. The membrane of the upper chamber was either uncoated or coated with collagen IV. The FS cells are grown in the bottom of the well either directly on the plastic, on a thin film of human placental extra-cellular matrix (ECM), or inside 3D Matrigel. Results: First we explored the influence of different ECM substitutes in monocultures of FS cells. The effect of the 2D human ECM substitute was variable, whereas the 3D Matrigel consistently resulted in 3-4 fold increase of uPA mRNA. Then we tested different cancer cells in combinations with the FS cells. Only certain cell combinations resulted in increased expression of uPA mRNA. The “best” combinations resulted in a 2–3 fold increase of uPA mRNA. The type of cancer cells, the BM substitute for the cancer cells, and the ECM substitute for the FS cells influenced the response in FS cells. These variable results are taken to indicate a complex crosstalk between the cell types, regulated expression of paracrine signals in the cancer cells, and probably regulated expression of receptors in the FS cells. The content of uPA mRNA in cancer cells was reduced by collagen IV coating in HEY cells but not in ES-2 cells. Presence of FS cells embedded in Matrigel in the lower chamber had variable effects. In contrast, Matrigel without FS cells in the lower chamber had no effect. Induction of uPA mRNA by ES–2 cells could be reproduced by incubating FS cells, embedded in Matrigel, with ES–2 cell conditioned medium, and in this system we employed neutralizing antibodies to 10 different growth factors/cytokines, which reportedly induce uPA. We found that antibodies to bFGF and EGF, added to the conditioned medium, reduced the uPA mRNA induction by 20% each at 12 hours. Conclusions: The crosstalk between cancer cells and SF cells is complex, and regulation of uPA gene expression varies with combination of cell types. ES–2 cells, which derive from a poorly differentiated ovarian cancer, are by far the most fast growing cell line tested, and are powerful inducers of stromal uPA mRNA. The inducing signal from ES–2 cells includes bFGF and EGF.

Keywords: Ovarian cancer, uPA mRNA, stroma
Tumor Cell Signaling Through Integrin alpha5beta1 Requires Urokinase Receptor Binding

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Upregulation of urokinase receptors (uPAR) is common during tumor progression and thought to promote metastasis. uPAR directly binds urokinase (uPA) and a set of beta1 integrins, but it remains unclear to what degree uPAR/integrin binding is important to beta1 integrin signaling. Using site-directed mutagenesis we have identified single amino acid residues in uPAR domains 2 and 3 that fail to associate with either alpha3beta1 (D262A) or alpha5beta1(H249A) but associate normally with uPA. To study the effects of uPAR mutations on beta1 integrin function, we first stably silenced uPAR by expression of hairpin RNAi in multiple tumor cell lines: HT1080, H1299, and H838. Knockdown of uPAR in tumor cells resulted in markedly reduced fibronectin (Fn) and alpha5beta1–dependent ERK phosphorylation, MMP9 expression, and lamellipodia formation. UPAR cDNAs were then constructed that were insensitive to RNAi knockdown and used to express wild type or mutant uPars in knockdown cells. Re-expression of wt or D262A mutant uPAR but not the non-alpha5beta1 binding H249A uPAR reconstituted Fn responses. Because uPAR/alpha5beta1 binds Fn in exons 12–14 whereas alpha5beta1 binds in exons 9–11, we tested whether signaling and MMP9 responses were binding site dependent. Only cells engaging both Fn exons 9–11 and exons 12–14 activated ERK and upregulated MMP9. Thus uPAR is required for maximal alpha5beta1–dependent responses to Fn and this operates through enhanced signaling following integrin attachment to separate sites on Fn by alpha5beta1 and uPAR–bound alpha5beta1. These findings provide a direct link between uPAR expression and the metastatic phenotype.

Keywords: urokinase receptor, integrin, fibronectin
Gene and protein expression profiling of human MMPs and TIMPs in primary tumors derived from high and low disseminating variants of HT-1080 fibrosarcoma

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Proteolytic modifications of the extracellular matrix proteins and cell surface receptors by matrix metalloproteinases (MMPs) are critically important for tumor cell dissemination and metastasis. Functional activity of MMPs is tightly regulated through gene and protein expression as well as the degree of proenzyme activation and inhibition of the activated enzyme by natural tissue inhibitors of metalloproteinases (TIMPs). A great deal of information has accumulated regarding the role of MMPs and TIMPs in tumor cell invasion and tumor-induced angiogenesis. However, only limited data are available on the role of the MMP/TIMP system in tumor cell intravasation, although it is one of the rate-limiting steps in the metastatic cascade. To analyze intravasation of tumor cells, we have generated two variants of human HT-1080 fibrosarcoma, which differ 100-fold in their ability to enter the vasculature of the chorioallantoic membrane (CAM) and metastasize to the secondary organs of the chick embryo. The availability of these high (HT-hi/diss) and low (HT-lo/diss) disseminating cell variants allowed us to investigate the role of MMPs and TIMPs specifically in tumor cell intravasation. By quantitative PCR and Western blot analyses, HT-hi/diss and HT-lo/diss cells were initially profiled for in vitro mRNA and protein expression levels of nine MMPs (MMP-1, -2, -3, -7, -8, -9, -10, -13, and -14) and three TIMPs (TIMP-1, -2, and -3), known to be expressed in the parental HT-1080 cells. To delineate tumor and host MMPs and TIMPs, which are actually involved in tumor cell intravasation, in vivo mRNA and protein profiling analyses were performed on CAM primary tumors derived from the two HT-1080 cell variants. Human MMP-1 and MMP-9 were more abundant in HT-hi/diss variant than in HT-lo/diss variant both in cell cultures in vitro and in primary tumors in vivo. In contrast, human MMP-2 and TIMP-2 were consistently expressed at higher levels in the HT-lo/diss cells and tumors. To further assess the role of MMP/TIMP system in tumor cell intravasation, activity based protein profiling (ABPP) of the two HT-1080 cell variants was performed with a metalloprotease-specific probe. Surprisingly, ABPP indicated only minor differences in MMP activity between the two HT-1080 cell variants. This discrepancy between results of the different profiling methods could indicate that many of the tumor MMPs are functionally inactive due to complex formation with TIMPs. Functional roles of human TIMP-1, -2 and -3 in intravasation of HT-1080 cell variants were evaluated by downregulation of protein expression with the respective siRNA. Our findings on downregulation of individual TIMPs and their combinations indicate that a fine balance between tumor MMPs and their natural inhibitors TIMPs might determine success of an intravasation event during tumor cell dissemination.

Keywords: Metastasis, matrix metalloproteinase, TIMP
The ratio of Matriptase/HAI-1 mRNA is higher in colorectal cancer adenomas and carcinomas than corresponding tissue from control individuals.

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Purpose: It has recently been shown that overexpression of the serine protease, matriptase, in transgenic mice causes a dramatic increased frequency of carcinoma formation. Overexpression of HAI-1 and matriptase together changed the frequency of carcinoma formation to normal. This suggests that the ratio of matriptase to HAI-1 influences the malignant progression rather than the expression level per set. Dysregulated matriptase/HAI-1 ratio has been shown to strongly potentiate chemical carcinogenesis. This aim of this study has been to determine the ratio of matriptase to HAI-1 mRNA expression in affected and normal tissue from individuals with colorectal cancer adenomas and carcinomas as well as in healthy individuals, in order to determine whether a dysregulated ratio of matriptase/HAI-1 mRNA is present during carcinogenesis.

Experimental design: Using RT-PCR, we have determined the mRNA levels for matriptase and HAI-1 in colorectal cancer tissue (n=9), severe dysplasia (n=15), mild/moderate dysplasia (n=21) and in normal tissue from the same individuals. In addition, corresponding tissue was examined from healthy volunteers (n=10). Matriptase and HAI-1 mRNA levels were normalized to beta-actin. Results: Matriptase levels showed a modest down-regulation in carcinomas, whereas HAI-1 expression was extensively down-regulated in tissue displaying mild/moderate dysplasia and in normal and affected tissue from individuals with severe dysplasia or carcinomas.

Conclusions: Both colorectal adenomas and carcinomas displayed a higher matriptase/HAI-1 mRNA ratio than corresponding normal tissue from the same individuals or healthy controls. This shows that dysregulation of the matriptase/HAI-1 mRNA occurs early during carcinogenesis and is maintained during all stages of malignant progression.

Keywords: matriptase, HAI-1, cancer
Distinct alterations in the expression levels of SRA1 gene, in the breast cancer cell line MCF7, as a response to treatment with anticancer drugs.

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SR proteins is a group of highly conserved proteins in metazoans, that are required for constitutive splicing, influencing alternative splicing regulation. There is highly increasing evidence that many SR protein splicing factors are involved in cancer pathobiology through their effect on alternative processing decisions. SRA1 gene is a new member of the SR proteins, identified and cloned by members of our group, which appears to be the human homologue of the rat A1 gene. The SRA1 gene is located on chromosome 19, between the apoptotic gene BCL2L12 and the RRAS oncogene and encodes for a human high–molecular weight SCAF protein. It has also been demonstrated from members of our group that SRA1 is constitutively expressed in most of the human tissues, while it is overexpressed in a subset of ovarian tumors. Our previous results have suggested that SRA1 is associated with cancer progression and may possibly be characterized as a new marker of unfavorable prognosis for ovarian and breast cancer. In order to investigate the significance of the SRA1 expression in prediction of chemotherapy response in breast cancer, we studied the possible alterations in its mRNA expression levels, as a response to MCF-7 cells treatment with distinct anticancer drugs including cisplatin, carboplatin, doxorubicin, etoposide and taxol. The kinetics of cell toxicity were evaluated by the MTT method, whereas the expression levels of the SRA1 gene were analysed by RT-PCR, using gene specific primers. The percentage of non–viable cells was upregulated with increasing concentrations and cell exposure time to anticancer drugs. Distinct modulations of the SRA1 gene, at the mRNA level, were also observed. More specifically, the mRNA levels of the novel splicing–related gene SRA1 were down–regulated after treatment of MCF–7 cells with either cisplatin, carboplatin, doxorubicin or taxol, but remained in control levels upon treatment with etoposide. Acknowledgements: The present study was funded through the grant ‘KAPODISTRIAS’ of the University of Athens.

Keywords: Breast Cancer, MCF-7, SR proteins
Real-time PCR analysis of BCL2L12, a novel member of BCL2 family of the apoptosis-related genes, in human leukemia.

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Modulations in the expression levels of the BCL2 gene network have been implicated in the pathogenesis of distinct types of leukemia. Recently, a new member of the BCL2 family of the apoptosis related genes, BCL2L12, was cloned by members of our group. BCL2L12 maps to chromosome 19q13.3 and is located between the IRF3 and PRMT1 genes, close to RRAS oncogene. Also, according our recent study, important modulations in the mRNA levels of the gene were observed in HL-60 leukemia cells under treatment with either cisplatin, carboplatin or doxorubicin, depending on both the apoptosis inducer and the specific apoptotic pathway induced, implying a direct relation between BCL2L12 mRNA alterations and apoptosis. In the present study we investigated the expression of BCL2L12 in 15 blood samples from patients with acute promyelocytic leukemia and 15 blood samples from apparently healthy people. High sensitive quantitative real time RT-PCR (QRT-PCR) was performed using SYBR Green method on a thermal cycler ABI Prism 7500. Total RNA was extracted from cells using TRI Reagent and 2 micrograms were reverse–transcribed into first-strand cDNA. Based on the information of the BCL2L12 gene sequences, two pairs of gene–specific primers were designed. cDNAs from BCL2L12 were amplified using regular and quantitative real time PCR. GAPDH was used as housekeeping gene. BCL2L12 was found be expressed higher in leukaemia samples than bloods from apparently healthy people. Further work is ongoing in order to ascertain whether the mRNA expression levels of BCL2L12 may present a significant relationship with prognosis prediction in human leukemia. Acknowledgements: The project is co–funded by the European Social Fund and National Resources –(EPEAEK II) PYTHAGORAS II.

Keywords: leukaemia, BCL2, BCL2 L12
Kallikrein-related (KLK) serine proteases and their roles in prostate cancer: Nuclear, cytoplasmic and secreted forms.

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The human tissue kallikrein–related (KLK) proteases are a multigene family of 15 serine proteases, many of which are over–expressed in prostate cancer. Prostate–specific antigen (PSA), a member of this family, is the current serum biomarker for detection and monitoring of prostate cancer. The related prostatic protease, KLK2/KLK2 is also emerging as an additional biomarker for prostate cancer. We have demonstrated that KLK4/KLK4 may also be a useful biomarker for prostate cancer as KLK4/KLK4 expression is higher in the pre–malignant prostatic intra–epithelial neoplasia (PIN) lesion, adenocarcinoma and bone metastasis compared to benign/normal glands. Of interest, there are over 70 KLK splice variants many of which are cancer–specific in their expression and which encode truncated KLK proteins that would not be catalytically active. At least one of these variants, KLK4–205, is nuclear localized which is a novel site for a serine protease–related protein. The functional role of this variant or the native PSA, KLK2 or KLK4 proteases in cancer progression are not fully elucidated, although several biochemical studies suggest a matrix degrading role either directly or indirectly via the activation of urinary plasminogen activator and other factors. In order to further demonstrate a functional role at the cell biology level, we have stably-transfected the prostate cancer PC3 cell line with prepro-PSA, –KLK2 and –KLK4. No change in the proliferative or invasive capacity of these transfected cells was observed, but PSA and KLK4, but not KLK2, over–expression elicited a morphological change and increased migration to various chemo–attractants. We also observed a loss of the cell adhesion protein, E–cadherin and down–regulation of a number of other cell adhesion molecules (desmplakin, junction plakoglobin, claudin–3 and claudin–7) while expression of the mesenchymal marker, vimentin, was increased. These changes are indicative of an epithelial to mesenchymal transition (EMT) and a more aggressive phenotype. KLK4:PC3 cells showed significantly greater attachment to collagen I and IV and increased migration towards conditioned media from an osteoblastic cell line (Saos–2). PSA and KLK4 expression was also increased in LNCaP:Saos 2 cell co–cultures. These data provide compelling evidence for a role for these kallikrein–related serine proteases in more aggressive disease especially bone metastasis and highlight their potential as biomarkers and therapeutic targets.

Keywords: kallikrein–related proteases, prostate cancer, nuclear and cytoplasmic forms
Clinical Aspects

**Japanese guidelines for pulmonary thromboembolism (PTE) prophylaxis is effective for a decrease in the occurrence of PTE**

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**Purpose:** Venous thromboembolism (VTE), which had been considered a relatively rare disease in Japan, has been on the increase in recent years as eating habits have become more similar to those of the West. The Ministry of Health, Labor and Welfare in Japan reported in a patient survey that there were 4,000 patients with pulmonary thromboembolism (PTE) and 1,738 deaths from PTE in 1999 increasing in about 3 times for a decade. We investigated the clinical efficacy of thromboprophylaxis for perioperative PTE after drafting Japanese guidelines.

**Methods:** The Japanese Society of Pulmonary Embolism Research analyzed 309 cases of acute PTE among a total of 533 registry patients. Main risk factors were recent major surgery, cancer, prolonged immobilization, and obesity; only a few patients had coagulopathy. Among 110 cases of recent major surgery, PTE occurred associated with orthopedic surgery (29.1%), general surgery (21.8%), gynecological surgery (18.2%), neurosurgery (8.2%), urological surgery (5.5%), and others (17.3%). In-hospital mortality rate was 14%. In patients with cardiogenic shock, the mortality rate was reduced by thrombolysis. Based on these investigations we drafted Japanese guidelines for perioperative PTE prophylaxis in 2004. And, perioperative PTE was investigated by Editorial Committee on Guideline for Prevention of Venous Thromboembolism by Japanese Society of Anesthesiologists from 2002 to 2004.

**Results:** The incidence of PTE in Japan is considered to be one level lower compared with Western populations according to ACCP (American College of Chest Physicians) guidelines. Furthermore, low molecular weight heparin (LMWH) is not covered by health insurance and is contraindicated for pregnant women still now in Japan. We classified four risk groups according to ACCP guidelines. Recommended thromboprophylaxis is early mobilization for low risk group, elastic stocking (ES) or intermittent pneumatic compression (IPC) for moderate risk group, IPC or low dose unfractionated heparin (LDUH) for high risk group, and LDUH + IPC or LDUH + ES for highest risk group. And, risk group should be raised one rank in cases with any additional risks, such as obesity, advanced age, pregnancy, operation time, and other complications. 369 cases of PTE were registered to Editorial Committee by Japanese Society of Anesthesiologists in 2002. The rate of perioperative PTE is estimated to be 0.044% (369/837,540), and the fatal rate among clinical PTE was 17.9%. 36% of the cases occurred in orthopedics, 22% in general surgery and 10% in obstetrics and gynecology. 59% of the cases did not received prophylaxis, and 52% of the cases were restricted mobility. The rate of perioperative PTE has increased to be 0.048% (440/925,260) in 2003, however, its rate has decreased to be 0.036% (409/1,131,154) in 2004. Fortunately, the management fee for PTE prophylaxis was established and covered by health insurance in April 2004. Surprisingly, the incidence of perioperative PTE decreased just after this guideline was issued.

**Conclusion:** Japanese guideline for PTE prophylaxis is effective for a decrease in the occurrence of PTE. After accumulation of further clinical evidences and application of pharmacological agents, such as LMWH and fondaparinux, we will establish the advanced guidelines in the future.

**Keywords:** pulmonary thromboembolism, thromboprophylaxis, Japanese guidelines
Quantification of soluble fibrin and D-dimer at the pregnancy with the risk of fetal loss

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Aims. The main purpose of the study was to obtain fibrin-specific monoclonal antibodies (mAbs), to carry out immunoenzyme methods for quantification of soluble fibrin and D–dimer in human blood plasma and to verify the methods at the pregnancy with the risk of fetal loss (RFL).

Methods. Usual hybridoma technique was used for production of mAbs. MAbs were isolated from cultural media by affinity chromatography on protein G- and fibrin- sepharoses. ELISA and Western–blot analysis were used to localize the epitopes for mAbs obtained. Results. Partly denaturated human fibrin desAABB was used for mice immunization. As a result three hybridomas have been obtained, which produce mAbs reacting with human fibrinogen without cross–reaction with fibrinogen. Competitive ELISA testified that epitopes for these mAbs in fibrin molecule practically coincided. ELISA and immunoblot analysis with cyanogen bromide and plasmin degradation products of fibrin showed that epitopes for these mAbs were situated in fibrin fragment Bbeta118–134. Thus this region is the neoantigenic determinant being exposed during fibrinogen–fibrin transformation. Fibrin–specific mAb 1A–5C was used in double-sandwich ELISA as a “catch”–antibody and our mAb II–4d – as a “tag”–antibody for soluble fibrin quantification. We obtained earlier D–dimer–specific mAb III–3b and showed that epitope for this mAb in human D–dimer was localized in Bbeta155–160 fragment. It has been shown that mAb III–3b reacts also with oligomeric plasmin degradation products of fibrin. Thus Bbeta155–160 region is the neoantigenic determinant, being exposed during fibrin degradation by plasmin. MAb III–3b was used in double–sandwich ELISA as a “catch”– antibody and our mAb II–4d – as a “tag”–antibody for D–dimer quantification. There are a lot of data on the direct relation between the RFL and thrombophilia. So this complication of pregnancy may be accompanied by the increase of soluble fibrin and D–dimer quantity. We performed quantifications of soluble fibrin and D–dimer in blood plasma of healthy pregnant women (n = 33) and pregnant women with RFL (n = 44). D–dimer concentrations varied in the range of 1 – 224 ng/ml at the pregnancy period from 4 till 37 weeks. There was positive correlation between D–dimer concentration and pregnancy term both at normal pregnancy and pregnancy with RFL. The mean values of D–dimer concentration at various terms of normal pregnancy and pregnancy with RFL did not vary considerably. The increased mean values of soluble fibrin concentrations were observed at pregnancy with RFL as compared to the normal pregnancy at the terms from 4 till 24 weeks (17,87 mkg/ml and 9,03 mkg/ml correspondingly, P less 0,05).

Conclusions. Three fibrin–specific mAbs have been obtained with epitopes being localized in fibrin fragment Bbeta118–134. One of these mAbs was used in double–sandwich ELISA as a “catch”–antibody for soluble fibrin quantification in human blood plasma. Soluble fibrin quantification proved to give useful diagnostic information at the pregnancy with the risk of fetal loss.

Keywords: soluble fibrin, D–dimer, the risk of fetal loss
The repeatability of quantitative D-Dimer assays in clinical laboratories

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Nowadays, assessment of D-Dimer is widely used for the exclusion of venous thrombosis. For the proper use of cut-off levels information about the analytical performance of D-Dimer test should be known. Recently the within- and between-laboratory variation of quantitative D-Dimer assays was investigated (1). Here we studied the repeatability of quantitative D-Dimer assay using data of the external quality assessment programme of the ECAT Foundation. In 2005, three different plasma samples with a D-Dimer level close to the cut-off level were distributed twice to the participants in different surveys. The repeatability was defined as a coefficient of variation (the difference between the measurements divided by the mean value of both measurements). There is an average repeatability of 8.4% (range: 7.3 – 10.5%, n=156–178). The average repeatability for different methods is comparable, notably for the Vidas (bioMerieux) method 7.2% (range: 6.3 – 8.3%, n= 47–62), for the AutoDimer (Trinity) method 8.0% (range: 6.5 – 9.2%, n=13–15), for the Tinaquant (Roche Diagnostics) method 7.6% (range: 6.2 – 9.9%, n=37–41) and for the STA Liatest (Diagnostica Stago) 9.4% (range: 6.5 – 13.4%, n=40–43). Based on the observed repeatability the 95%-range of uncertainty around the cut-off level was calculated. This is for the Vidas method 428 – 572 ng/mL, for the AutoDimer method 151 – 209 ng/mL, for the Tinaquant method 424 – 576 ng/mL and for the Liatest 406 – 594 ng/mL. Samples with a test result above the upper limit of the uncertainty range can be defined as positive, while test results below the lower limit of the uncertainty range can be defined as negative. Samples with a test result within the uncertainty range should be repeated before a proper clinical decision can be made. When the average of both measurements is below or above the cut-off level the sample can be defined as negative or positive, respectively. (1) M. Spannagl, F. Haverkate, H. Reinauer, PO. Meijer. Blood Coag and Fibrinol 2005; 16: 439 – 443.

Keywords: D-Dimer, Cut-off level, Uncertainty range
Fibrin degradation by various enzymes: consequences for D-dimer tests

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Degradation of fibrin is not only possible by plasmin, but also by leukocyte enzymes (Elastase, Cathepsin G) and Metalloproteinases (e.g. MMP-3, MMP-7, MT1-MMP). To evaluate whether or not the degradation products were recognised by currently used D-dimer assays we prepared lysates from fibrin clots prepared from diluted plasma or purified fibrin. Both clots with and without crosslinking by factor XIII were lysed. Fibrin clots were prepared in microtiter wells and lysis was induced by the enzymes added on top of the preformed fibrin, or included into the clot before coagulation. We obtained lysates from complete lysis by t-PA, u-PA, Streptokinase, Plasmin, PMN-elastase, MMP-3 and MMP-12. MMP-9 showed no lysis; Lysis by MMP-12 was not reported before. Analysis was done with assays for D-dimer (FbDP, Vidas, STA-LIA, Asserachrom, Tinaquant) and for elastase degradation products of fibrinogen (FgEDP). Most clear results obtained were for (a) FgEDP which was specific for fibrinogen degradation by elastase, and showed no reaction with any fibrin degradation lysate including that from PMN-elastase. (b) Asserachrom showed only signals with plasmin or plasminogen activator induced clot lysis (c) FbDP showed a similar specificity as Asserachrom with the exception of direct lysis by preformed plasmin (d) STA-LIA and Tinaquant showed clear reactions (10-20% signal relative to t-PA-plasmin degradation products) with elastase, MMP-3, and MMP-12 degradation products. It is concluded that degradation products from different enzymes are recognised differently by various assays and that potentially the use of enzyme-specific assays is a method for differential diagnosis related to the origin of fibrin degradation products.

Keywords: D-dimer, elastase, Metalloproteinases
Products of limited elastase/cathepsin G degradation of fibrinogen as new biomarker in cardiovascular disease

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A new biomarker assay specific for leukocyte elastase/cathepsin G– proteolytic damage of fibrinogen (FgEDP) has been evaluated. In healthy controls, plasma values show a stable level with a coefficient of variation between and within individuals of 29 and 9 %, respectively. We evaluated the levels in type II diabetics before and during treatment with atorvastatin and in a prospective study on outcome in unstable angina pectoris patients. Levels of FgEDP in 170 type II diabetics without cardiovascular complications tested before treatment with atorvastatin were about 1.5 times higher than in age-matched healthy controls. Principle component analysis showed a relationship between FgEDP and the acute phase proteins C–reactive protein (CRP), fibrinogen, serum amyloid A and s–phospholipase A2. There was also a relationship of increased FgEDP with intima media thickness (p=0.018 in males, tendency in females). Treatment with 10 mg atorvastatin for 30 weeks reduced FgEDP levels with 4.9% (n.s., n=57) and 9.1% with 80 mg atorvastatin (p=0.034, n=56) Levels in 211 unstable angina pectoris patients upon admittance to hospital were also about 1.5 times higher than levels in healthy controls, and showed a very close relationship with the inflammation markers CRP, fibrinogen and interleukin–6. Patients with a poor outcome showed an odds ratio of 3.84 (95% CI: 1.44–10.3) for FgEDP. This OR was larger than for CRP (1.92) and fibrinogen (2.98). It was noted that specifically a combination of elevated FgEDP and CRP showed superadditive prediction. It is concluded that a proteolytic foot–print of elastase–cathepsin–G proteolytic damage of fibrinogen provides for a stable plasma biomarker, which is increased in conditions of cardiovascular risk, can be reduced by statin treatment and shows predictive capacity for clinical outcome.

Keywords: fibrinogen, proteolytic footprint, Diabetes
Treatment with tranexamic acid (TA) of patients with mild bleeding disorders: Prolongation of clot lysis time is closely related to plasma levels of TA

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Tranexamic acid (TA), a synthetic derivative of lysine, inhibits fibrinolysis through blockade of lysine binding sites on plasminogen. TA is empirically used with some success in patients with mild bleeding disorders. However, there are no studies relating the TA levels to clot lysis time (CLT), procarboxypeptidase U (TAFI) plasma concentrations and bleeding time (BT). We assessed these variables, including plasminogen levels (Pg), endogenous thrombin generation (ETG) in platelet rich plasma, and plasma levels of uPA, tPA and PAI-1 in 48 patients (18±12 years; 33 female/15 male) with mucocutaneous bleeding (von Willebrand disease (VWD) =7, platelet function defects (PFD)= 9, VWD+PFD= 1, and patients with bleeding of unknown cause= 31). TA was orally administered (15–25mg/kg, tid, for 3 days). Determination of plasma TA concentrations (liquid chromatography–tandem mass spectrometry method) and the other variables were performed before and after intervention. The mean ± SD of TA concentration in plasma at the end of treatment was 1.5 ± 1.8 µg/mL and these levels were significantly correlated (r=0.8929, p<0.0001) with the prolongation of CLT measured at the same time (316 ± 382 vs 54 ± 25 min, p<0.0001) TA intake resulted in no significant changes in plasma uPA, tPA and PAI-I antigens. Also, changes in plasma VWF antigen/function and platelet aggregation/secretion were not significant. TA intake resulted in a significant decrease in plasminogen levels: 87 ± 26 % vs 101 ± 26 % (p<0.0001, paired “t” test) and significant increases in proCPU levels (716 ± 99 vs 669 ± 91 U/L, p<0.0001) and ETG (1544 ± 312 vs 1453 ± 311 nM thrombin, p<0.03). Conclusions: The hemostatic action of TA is explained by a dramatic prolongation of CLT, a slight increase in proCPU and a concomitant decrease in Pg and increase in ETG. Measurement of CLT, an easily performed test, appears to be a good surrogate assay to monitor the effect of TA when plasma TA cannot be measured. The relationship between the clinical effect of TA (bleeding arrest) with CLT and plasma TA levels needs to be assessed. This information should be of great usefulness for monitoring antifibrinolytic therapy, especially in non-responder bleeders.

Keywords: tranexamic acid, clot lysis, bleeding disorders
Antibodies to tissue-type plasminogen activator (t-PA) in patients with inflammatory bowel disease

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Background: Patients with inflammatory bowel disease (IBD) have an increased prevalence of thromboembolic events. The pathogenetic mechanisms of these events include reduced fibrinolysis, which may be caused by antibodies to tissue-type plasminogen activator (t-PA).

Objectives: To evaluate anti-t-PA antibodies in patients with IBD, considering clinical, biochemical and functional characteristics. Patients and methods: We immunoenzymatically measured anti-t-PA antibodies in plasma from 97 consecutive IBD patients and 97 ageand sex-matched healthy controls. We also assessed the antibody interactions with different epitopes of t-PA, the antibody inhibition on t-PA activity and the correlations with clinical features and other serum antibodies.

Results: IBD patients had higher median anti-t-PA antibody levels (5.4 U/mL vs. 4.0 U/mL; P<0.0001): 18 patients were above the 95th percentile of the controls (OR 5.3; 95% CI 1.7–16.3; P<0.003), and the six with a history of thrombosis tended to have high levels (6.9 U/mL). Anti-t-PA antibody levels did not correlate with IBD type, activity, location or treatment, or with age, sex, acute-phase reactants or other antibodies. The anti-t-PA antibodies were frequently IgG1 and bound t-PA in fluid phase; they recognized the catalytic domain in 10 patients and the kringle-2 domain in six. The IgG fraction from the three patients with the highest anti-t-PA levels slightly reduced t-PA activity in vitro.

Conclusions: The prevalence of anti-t-PA antibodies is high in IBD patients. By binding the catalytic or kringle-2 domains of t-PA, these antibodies could lead to hypofibrinolysis and contribute to the prothrombotic state of IBD.

Keywords: tissue type plasminogen activa, antibodies, inflammatory bowel disease
The clinical significance of hyperfibrinolysis in onset of disseminated intravascular coagulation syndrome

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Well known that disseminated intravascular coagulation (DIC) syndrome initiated as a result of disbal-ancement between the coagulation and fibrinolysis. The thrombin that represent the coagulation system, and plasmin that represent the fibrinolytic system, appear to be the key mediators of DIC syn-drome (Craig, 2004). The activation of fibrinolysis in procoagulation shift is the compensatory effect for restoration of vessel permeability and prevention of multiorgan disfunction (Asakura H. et al., 2001), but the general hyperactivation of fibrinolysis may induce the pathogenesis of DIC by itself, result in damage of coagulation factors and coagulopathy of consumption. In investigation of 1286 patients with the DIC syndrome, T. Matsuda (1996) mark out two clinic–laboratory variants of DIC syndrome: fibrinolytic–dominant DIC and coagulation–dominant DIC syndrome. Regardless of common opinion of secondary role of hyperactivation of fibrinolysis, it cause of initiate of DIC syndrome in half of cases. The goal of study: learn the occurency of fibrinolytic variant of DIC syndrome after the pro-longed abdominal surgery. Materials and methods. Retrospective analysis of 630 patients was per-formed. The patients stays in intensive care unit in 2005 year after the prolonged (more than 6 hous) abdominal surgery. Eighty four patients had the different stages of systemic inflammatory processes (Bone R.C. et al., 1992): systemic inflammatory response syndrome in 40 patients, sepsis in 26 patients, and severe sepsis in 18 patients. The main laboratory tests of coagulation and thromboelas-tography performed daily. Based on the results of tests perform the differential diagnostic of revealed coagulopathies. Results and discussion. The most common coagulopathy, discovered in early postop-erative period was the fibronolytic variant of DIC syndrome – 20.8% of patients. The incidence of other coagulopathies was: the coagulation variant of DIC syndrome – 8.6%, thrombocytopenia/thrombocytopathia – 4.1%, coagulopathy as a result of hepatic failure – 2.9%, coagulopathy of haemodylu-tion – 2.4%, the overdose of direct anticoagulants – 1.9%, coagulopathy of consumption in DIC syn-drome – 0.3%. the hyperactivity of fibrinolysis characterized by the laboratory signs – increase of products of degradation of fibrinolysis more than 40 mg/ml, little prolongation of protrombine time, decrease of fibrinogen level, forming the porous clot depend on thromboelastogramme, and by the clinical signs in increased drainage secretion in the half of patients. The most frequent coagulopathy, displayed in SIRS is the fibrinolytic variant of DIC syndrome (37.5% of patients), so in sepsis and severe sepsis – 30.7% and 33.4%, correspondingly. The revealed trend explained by the pathogenetic mechanisms of development of sepsis. The initiate of fibrinolysis in early stages of inflammatory process occur parallel to the activation of coagulation, because both the systems has the similar trig-ger mechanisms by the inflammatory mediators (Asakura H. et al., 2001). In the same time during the increase of expression of inflammatory process take place the exhaustion of fibrinolytic part. Conclusion. The most frequent coagulopathy, revealed after the prolonged surgery is the fibrinolytic variant of the DIC syndrome.

Keywords: DIC syndrome, SIRS, surgery
Effect of APC resistance associated with factor V mutations on plasma fibrinolysis

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Factor V is endowed with both procoagulant and anticoagulant properties. APC resistance caused by homozygous factor V Leiden mutation has been shown to inhibit fibrinolysis through the enhancement of thrombin-mediated activation of TAFI (thrombin activatable fibrinolysis inhibitor). It is unclear, however, whether heterozygous carriers of Leiden mutation or carriers of other factor V genotypes associated with APC resistance have a defective fibrinolytic activity. We studied 107 subjects with different factor V genotypes. Sixty-seven were unrelated asymptomatic subjects with (n=34) or without (n=33) Leiden mutation while 40 belonged to 6 thrombophilic families with different combinations of factor V mutations (Leiden mutation, HR2 haplotype, and type I factor V deficiency). Plasma fibrinolytic capacity was studied by evaluating the lysis time of tissue factor-induced clots exposed to 25 ng/ml exogenous t-PA. The assay was performed in the absence and in the presence of APC (1 microg/ml) and the fibrinolytic response was calculated by the ratio of the two lysis times (APC-LR). Heterozygous Leiden mutation was associated with a significantly reduced APC-LR both in unrelated subjects (1.57 ± 0.26 as compared to 1.75 ± 0.26 in normal subjects, p<0.05) and in members of thrombophilic families (1.21 ± 0.22 as compared to 1.57 ± 0.29 in family members with no factor V mutations, p<0.05). Moreover, the combination of Leiden mutation with type I factor V deficiency (pseudohomozygous APC resistance) made the plasma virtually unresponsive to the fibrinolytic effect of APC (APC-LR: 1.1 ± 0.09, n=7), mimicking the homozygous Leiden mutation. Neither the HR2 haplotype nor type I factor V deficiency, when present in heterozygous form, influenced the response to APC to a significant extent. Interestingly, the two patients with Leiden plus HR2 haplotype and the patient with HR2 haplotype plus type I deficiency were also markedly refractory to the fibrinolytic activity of APC (APC-LR of 1.08 and 1.1, respectively). These data indicate that heterozygous carriers of the Leiden mutation are resistant to the fibrinolytic effect of APC. This resistance, however, is attenuated by the presence of normal factor V as indicated by the strong refractoriness to APC of the combinations of Leiden mutation with either type I deficiency or HR2 haplotype. These results may help understand the increased thrombotic risk associated with pseudohomozygous APC resistance or with the combination of HR2 haplotype with either Leiden mutation or type I factor V deficiency.

Keywords: Factor V, Protein C, Clot lysis
Performance of a new rapid quantitative D-dimer assay (HemosIL D-dimer Plus) in the diagnosis of pulmonary embolism. Comparison with other quantitative assays. Preliminary results

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The measurement of the specific fibrin degradation products D–dimer is widely used in the diagnostic work–up of pulmonary embolism (PE) and venous thromboembolism in general. Due to the heterogeneity of D–dimer assays, it is now recommended that the performances of each new D–dimer assay have to be evaluated in clinical trials. The HemosIL D–Dimer HS assay (Instrumentation Laboratory, Paris, France) is a new rapid quantitative latex–microparticle agglutination–based assay which is automated on the ACL TOP analyzer (Instrumentation Laboratory). Latex microparticles were sensitized with the F(ab')² fragment of a monoclonal antibody directed against D–dimer. The change in optical density at 671nm was recorded after addition of the plasma sample (16µL) and test result is usually obtained within 4 minutes. Test result is expressed in ng/mL (D–dimer) and the reference range in the normal population was 0–243 ng/mL as defined by the reagent manufacturer. Its within run precision (n=20) and its between run precision (n=8) were found to be below 7.0% using commercially available lyophilized plasma samples both in the subnormal and abnormal ranges of concentrations. The aim of the present study was to evaluate the performances in the diagnosis of PE and to compare with those of two other automated quantitative assays i.e. the HemosIL D–dimer assay (Instrumentation Laboratory) and the VIDAS D–dimer Exclusion assay (BioMérieux, Marcy l’Etoile, France). All three assays were performed according to the recommendations of the manufacturers, and the cut–off levels used were those reported in the literature and/or the package inserts of the assay kits i.e. 230 ng/mL for the HemosIL D–Dimer HS and the HemosIL D–Dimer assays and 500 ng/mL (FEU) for the Vidas D–Dimer Exclusion assay. We prospectively studied consecutive outpatients with chest pain and suspected PE (low and moderate pre–test probability) referred to the emergency ward of our Institution (University hospital). The preliminary results presented here corresponded to those of 166 patients referred during a 3–month period. The diagnosis of PE was retained in 12 cases (7.2%). The result of the 3 tested assays was found above the clinically defined cut–off level in all of these 12 patients with PE, leading to a sensitivity and a negative predictive value (NPV) of 100%. The result of the HemosIL D–Dimer HS assay was found to be above the cut–off level in 111 of the 155 patients without PE (72%). Similarly, D–dimer levels were found above the cut–off in 76% of these patients when evaluated with the HemosIL D–dimer and in 75% with the Vidas D–dimer Exclusion assay. The high proportion of elevated D–dimer levels in our series was related to the demographical characteristics of the studied population (mean age=72.5 years, range: 23–96). The specificity of the HemosIL D–Dimer HS assay was found to be higher than those of the two other quantitative assays. In conclusion, the HemosIL D–dimer HS assay allowed an accurate diagnosis of all of the patients with PE and so, could be validly used to exclude the diagnosis of PE in the case of a negative result (NPV=100%).

Keywords: D–dimer, pulmonary embolism, exclusion

* selected for oral presentation
† not represented at ISFP Congress
Emerging Technologies

**Homogeneous, Bioluminescent Assays for Proteasome Activity and Other Protease Therapeutic Targets**

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Protein degradation is mediated predominantly through the ubiquitin–proteasome pathway. The importance of the proteasome in regulating degradation of proteins involved in cell cycle control, apoptosis, and angiogenesis led to the recognition of the proteasome as a therapeutic target for cancer. The first generation proteasome inhibitor, bortezomib, is now an approved drug for the treatment of refractory multiple myeloma. Peptide-conjugated fluorophores are widely used as substrates for monitoring proteasome activity, but fluorogenic substrates can exhibit significant background and can be problematic for screening due to interference from test compounds. To address these issues, we developed a bioluminescent method that combines peptide-conjugated aminoluciferin substrates and a stabilized luciferase in a coupled-enzyme, homogeneous format. We first developed this method for monitoring caspase activities and have now developed homogeneous, bioluminescent assays for all three proteasome activities. We use Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin, and Z-nLPnLD-aminoluciferin to monitor the chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome, respectively. The luminogenic proteasome assays showed increased dynamic range and improved detection sensitivity compared to the analogous fluorogenic assays. In addition, the luminogenic assays rapidly attained maximum signal and held stable light emission, allowing for assay flexibility. We have applied this technology to a cell-based proteasome assay using the Suc-LLVY-aminoluciferin substrate in combination with a selective membrane permeabilization step. The cell-based assay does not require lysate preparation and enables measurement of proteasome activity in a more physiologically relevant environment. The assay has been tested on numerous cell lines and has been shown to monitor activity that is inhibited by the proteasome inhibitors, lactacystin and epoxomicin. The proteasome assays are designed in a simple “add and read” format and have been tested in 96 and 384-well formats. The homogeneous, luminogenic protease assay format has also been tested with substrates for DPPIV, calpain, as well as several caspases. In all cases, the luminogenic assays are at least 10-fold more sensitive than fluorogenic assays using comparable substrates. The luminogenic assays are faster than fluorescent assays due to the coupled-enzyme format and the resulting “glow” kinetics. Typically, maximum sensitivity is reached within 10–30 min. In general, the bioluminescent, coupled–enzyme assay format offers a more sensitive and rapid protease assay ideal for high-throughput screening. Various applications will be highlighted, as well as limitations of the technology.

**Keywords:** luminescence, luciferase, proteasome
Non-invasive, optical imaging of specific cell surface proteolytic activity in single living cells

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We have generated a simple, sensitive, and non-invasive assay that uses non-toxic reengineered bacterial cytotoxins with altered protease cleavage specificity for the visualization of specific cell surface proteolytic activity in single living cells. The assay, which detects the cell surface proteolysis-dependent internalization of modified anthrax toxin-beta-lactamase fusion proteins using fluorogenic beta-lactamase substrates, was capable of specifically imaging endogenous cell surface furin, urokinase plasminogen activator, and metalloprotease activity. The specificity of imaging was demonstrated using protease gene-inactivated cells, small molecule protease inhibitors, and macromolecular protease inhibitors. The devised assay is highly versatile and was readily adapted for fluorescence microscopy, flow cytometry, and fluorescent plate reader formats. Furthermore, it can be tailored to detect the activity or inhibition of any cell surface protease for which a specific peptide substrate can be derived. Because the imaging assay measures the activity of a cell surface protease in its natural milieu and is easy to miniaturize, we hypothesized that it would be ideally suited for the identification of new classes of protease inhibitors that do not target the active site of a protease, but rather the "micro-environmental" factors required for the generation of a specific cell surface protease activity. This would include inhibitors that affect protease secretion, cell surface attachment, zymogen activation, susceptibility to endogenous inhibitors, shedding from the cell surface, and more. To test this proposition, we adapted the assay for fully automated quantitative high-throughput screening, using a 1536-well plate, seven microliter reaction volume, 2000 cells/reaction format. A preliminary titrated screen of 29,592 compounds for their capacity to inhibit furin cleavage-dependent internalization of the anthrax toxin-beta-lactamase fusion protein identified seven compounds with an IC50 < 1 micromolar (0.02% hit rate), demonstrating the feasibility of the approach.

Keywords: Imaging, Membrane proteolysis, Quantitative high throughput screening
Fibrinogen

Biochemical predictors of total mortality in unselected acute coronary syndromes patients treated according to contemporary guidelines – time-dependent change in prognostic value


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Background: There are several biochemical predictors of the outcomes in ST-elevation myocardial infarction (STEMI), as well as in non–STEMI and unstable angina (NSTEMI/UA) patients (pts) but particular predictors may change their prognostic value upon time. Aim: to prospectively compare the prognostic value of different biochemical parameters for short 30–day (s), medium 6-month (m) and long-term 1–year (y) total mortality prediction in unselected acute coronary syndromes pts treated in an university cardiologic centre with 24–hour invasive cardiology on–site.

Methods: One-centre, unselected 601 STEMI and 330 NSTEMI/UA pts registry from 2002–2003 years analysis with one-year follow-up, using multivariate logistic regression model. The predictive value of the model was assessed with the use of area under ROC curves. Model’s goodness–of–fit were checked by Hosmer–Lemeshow tests.

Results: Mean age in 931 pts was 63 years, 88% STEMI and 81% NSTEMI/UA pts were treated invasive-ly. S, m, and y total mortality were: 9.8, 12.5 and 14.1%, respectively. All biochemical data obtained on admission were included in the final model. Four biochemical serum markers: C–reactive protein (CRP), fibrinogen (F), creatinine (C) and INR index (measured prothrombin time/ normal prothrombin time) were identified as those independently increasing total mortality, when dichotomized to supra–median and infra–median groups with: 12.75 mg/l for CRP, 381 mg/dl for F, 0.96 mg/dl for C and 1.08 for INR as cutoff values. Statistically significant odds ratios for total mortality with their 95%confidence interval in different time perspectives were as follow for: s (CRP 4.45 [1.25–15.9]), m (CRP 4.43 [1.47–13.35]; F 3.67 [1.42–9.52]; C 2.75 [1.23–6.15]) and y (CRP 3.85 [1.40–10.6]; F 3.93 [1.60–9.68]; C 2.36 [1.1–5.07]; INR 2.11 [1.0–4.44]), respectively. No other biochemical markers – including troponins – turned out to be independent predictors of total mortality after one year in this registry. The constructed models (s,m,y) were characterized by both high goodness–of–fit and predictive value (ROC=0.72–79)

Conclusions: CRP>12.75 mg/dl, C>0.96 mg/dl, F>381 mg/dl and INR>1.08 measured directly on admission may predict total mortality after one year in unselected STEMI, NSTEMI/UA pts treated according to contemporary guidelines. F>381 mg/dl has the strongest predictive value in y follow–up, CRP>12.75 mg/dl is the only independent predictor for all: s, m, and y time perspectives.

Keywords: acute coronary syndromes, fibrinogen INR, prognosis
Lipoprotein(a) can alter fibrin clot permeability and susceptibility to lysis in vivo

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Lipoprotein(a) [Lp(a)] has been reported to be associated with increased risk of cardiovascular disease via several mechanisms, including inhibition of fibrinolysis. It is not known, however, whether elevated Lp(a) levels may affect clot properties in human subjects. In two groups: 52 apparently healthy men and 24 men with stable angina, who survived myocardial infarction (MI), aged 40 to 65 years, we determined fibrin clot permeation in a pressure-driven tube system, the kinetics of fibrin formation by turbidimetry, and fibrinolysis by measuring the fibrin assembly kinetics by spectrophotometry upon addition of recombinant tissue plasminogen activator. As expected, clot permeability was reduced and clot lysis was slower in MI patients as compared to healthy individuals. The median Lp(a) concentration was higher in patients than in healthy subjects (28.8 vs 13.2 mg/dL; p=0.002). In healthy individuals and MI patients with a cutoff of Lp(a) <30 mg/dL, fibrin clot permeation was higher (p<0.001) and fibrinolysis time shorter (p<0.05) by about 20% than that observed in subjects with Lp(a) >=30 mg/dL. Plasma Lp(a) showed associations with clot permeation (r=-0.67; p<0.0001) and lysis time (r=0.63; p<0.0001) in healthy subjects as well as in MI patients (r=-0.54; p=0.006; r=0.42; p=0.01; respectively). In regression analysis after adjusting for confounding factors, including age, fibrinogen, and CRP, Lp(a) represented an independent predictor of clot permeation (R= 0.61; p<0.0001 and R= 0.52; p<0.0001, respectively) and clot susceptibility to lysis (R=0.48; p=0.007 and R= 0.5; p=0.005, respectively) in healthy individuals and MI patients. Our results indicate that elevated plasma Lp(a) levels correlate with decreased fibrin clot permeation and impaired susceptibility to fibrinolysis both in apparently healthy subjects and patients with advanced coronary artery disease. Altered fibrin clot properties are likely a novel prothrombotic effect of Lp(a) in vivo.

Keywords: lipoprotein(a), fibrin, fibrinolysis
**HOMOCYSTEINYLATION OF HUMAN FIBRINOGEN LEADS TO FORMATION OF FIBRINOGEN-ALBUMIN CONJUGATES AND INCREASED RESISTANCE TO FIBRINOLYSIS**

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We have previously shown that hyperhomocysteinemic rabbits have altered fibrinogen function. This acquired dysfibrinogenemia is characterized by fibrin clots that are composed of abnormally thin, tightly-packed fibers with increased resistance to fibrinolysis. Homocysteine thiolactone is a metabolite of homocysteine (Hcys) that can react with primary amines and introduce a new sulfhydryl group. Recent evidence suggests that HCys thiolactone–lysine adducts form in vivo. We have recently demonstrated that reaction of Hcys thiolactone with purified fibrinogen (Hcys–fibrinogen) leads to properties that are similar to fibrinogen from homocysteinemic rabbits: 1) they both form clots composed of thinner, more tightly packed fibers than control fibrinogens; 2) the clot structure could be made more like control fibrin by increased calcium; 3) they both form clots than are more resistant to fibrinolysis than control fibrinogens. Mass spectrometric analysis of Hcys–fibrinogen revealed twelve lysines that were homocysteinylated. Several of these are in the alpha–C domain of fibrinogen, near the sites of two well-characterized mutations that are associated with thrombosis. In those mutations a cysteine replaces a non–cysteine reisude, thus introducing a new sulphydryl group. That free thiol mediates binding of a fraction of the circulating mutant fibrinogen to albumin. The incorporation of fibrinogen–albumin complexes into a fibrin clot results in thinner more tightly-packed fibrin fibers and increased resistance of the clots to fibrinolysis. We have found that HCys–fibrinogen also forms mixed disulfides with human albumin (HCys–Alb–fibrinogen). Fibrin clots containing HCys–Alb–fibrinogen show significantly impaired binding of tPA and a significant defect in tPA activation of plasminogen to plasmin. We have also found a high molecular weight form of fibrinogen in some human plasma samples that co-localizes with a high molecular weight form of albumin on Western blots. Thus, similar to cysteine-introducing mutations in fibrinogen, homocysteinylation mediates formation of fibrinogen–albumin complexes which might contribute to the prothrombotic state associated with homocysteinemia.

**Keywords:** fibrinolysis, homocysteine

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* selected for oral presentation

† not represented at ISFP Congress
Delaying Fibrinolysis with Synthetic Peptides Patterned on Fibrin B Knobs

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Recently we have reported that short peptides patterned on fibrin B knobs bind tightly to fibrinogen and change the onset of tPA-provoked fibrinolysis (Doolittle and Pandi, Biochemistry, 45:2657–67, 2006). The most potent of these peptides was GHRPYamide, which binds to human fibrinogen with an apparent dissociation constant of about 30 micromolar. The delays in fibrinolysis were paralleled by increases in the turbidity of clots, the opposite of expectation. The delay is not due to the turbidity per se, however, as evidenced by the fact that calcium ion also enhances clot turbidity but does not delay the onset of fibrinolysis. In the end, the delays were attributed to the synthetic peptides preventing the binding of the loosely tethered authentic B knob. We have extended these studies to other peptides, including alanine-ending knobs that bind exclusively to the betaC holes of fibrin(ogen). We have also studied the phenomenon in a lamprey system, the advantage of which is that the fibrinopeptide B can be released independently of the release of fibrinopeptide A. Our present interpretation of all the data is that the fibrinopeptide B can be released independently of the release of fibrinopeptide A. Our present interpretation of all the data is that the binding of the authentic B knob to the betaC hole traps the domain in a conformation which allows access to the tPA–plasminogen site at the distal end of the coiled coil (alpha chain residue Lys157), and the tightly binding synthetic peptides interfere. As is well known, the betaC and gammaC domains of vertebrate fibrinogen are descended from a common ancestor and at one point in time must have bound knobs of the same sequence. During the course of evolution the knob–hole involvements have differentiated to the point where there are now different jobs for the different knobs. The lamprey system allows some insight into the stages of this evolution.

Keywords: fibrin, fibrinolysis, synthetic fibrin knobs

* selected for oral presentation  † not represented at ISFP Congress
Plasma Fibrinogen Gamma Prime Chain Content in the Thrombotic Microangiopathy Syndrome

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PLASMA FIBRINOGEN GAMMA PRIME CHAIN CONTENT IN THE THROMBOTIC MICROANGIOPATHY SYNDROME Thrombotic microangiopathy (TMA) is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and microvascular thrombosis, a syndrome that includes thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). Human fibrinogen (‘fgn’) gamma’ chain variants contain a unique 20-residue C-terminal thrombin-binding sequence beyond residue 407, gamma’408–427. Full length gamma’ chains, gamma’1–427L, are constituents of a fibrin-dependent thrombin inhibitory system known as Antithrombin I, whereas post-translational, proteolytically processed fibrinogen gamma’ chains lacking the C-terminal EDDL427 sequence, termed gamma’423P, do not bind thrombin. In 87 healthy subjects (HS) and in 55 plasma samples from patients with TMA, we measured the total gamma’ chain–containing fibrinogen (TL gamma’–fgn):total fibrinogen (TL fgn) ratio and the full length gamma’ chain–containing fibrinogen (FL gamma’–fgn):TL fgn ratio. In HS plasmas, the TL gamma’–fgn:TL fgn ratio was 0.127 (i.e., 12.7% of the total fgn content), whereas the FL gamma’–fgn:TL fgn ratio, 0.099, was lower (p <0.0001). From the FL gamma’–chain:TL gamma’–chain ratios we calculated that non-thrombin-binding gamma’423P chains amounted to ~20% of the TL gamma’–chain content (range, 0% to 49%). In TMA plasmas, the TL gamma’–fgn:TL fgn ratio, 0.102, and the FL gamma’–fgn:TL fgn ratio, 0.085, were lower than their counterpart ratios in HS plasmas (p, <0.0001), whereas the proportion of truncated gamma’423P chains in TMA plasmas (~18%) did not differ significantly from HS values (p, 0.22). This suggested that the process leading to formation of gamma’ 423P chains in the TMA cohort does not differ from that in HS plasmas. Our findings suggest that in many cases of TMA, reductions in the content of FL gamma’–chains contribute to the microvascular thrombosis that characterizes TMA, possibly due to reduced Antithrombin I activity.

Keywords: fibrinogen, thrombotic thrombocytopenic pu, antithrombin
Identification of fibrinogen binding site of human histidine-rich glycoprotein (HRG)

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Human histidine–rich glycoprotein (HRG) is a 67kD plasma glycoprotein known to interact with fibrin(ogen), plasmin(ogen), et al. Previously, we showed that HRG enhances fibrinolysis by binding to fibrinogen (Abstr. No.OR325 of 20th ISTH Congress, 2005). Here, we report that HRG interacts with fibrinogen at the histidine–rich domain. [Methods] Fragmentation of HRG was performed, using reduced–pyridylethylated (RPe)–HRG, which was cleaved by CNBr, digested by lysylendopeptide, followed by further digestion by trypsin. Resulting peptides were separated by HPLC. Peptides with two and four repeats of Gly–His–His–Pro–His consensus sequence were synthesized by a peptide synthesizer. Interaction of HRG, its enzyme digestion–derived peptides or synthetic His–rich repeat peptides with fibrinogen was analyzed by an IAsys. [Results] 1) In the presence of zinc ion at a plasma concentration (20 micro M), HRG, in a concentration–dependent manner, enhanced the fibrinolytic activity of plasmin. 2) Interaction between HRG and fibrinogen was enhanced by 40–fold in the presence of the plasma level of zinc ion with the Kd of 3.2 x 10–8M. 3) RPe–HRG revealed essentially the same affinity for fibrinogen as native HRG, suggesting the binding is due to a local interaction of HRG with fibrinogen. 4) A fibrinogen–binding peptide with 10,147 Da was obtained by a lysylendopeptidase digestion. 5) Further degradation of the peptide by trypsin and 70% formic acid generated the smallest fibrinogen–binding peptide with 3327 Da, which was identified as that derived from Gln361 to His389 in the histidine–rich domain of HRG where the Gly–His–His–Pro–His repeat sequence is completely conserved. 6) Fibrinogen–binding ability of the Gly–His–His–Pro–His repeat was confirmed by the synthetic (His–His–Pro–His–Gly)4 peptide. [Conclusion] Taken together with our previous observations, we conclude that HRG enhances fibrinolysis by binding to fibrinogen at the histidine–rich domain in a zinc–dependent manner.

**Keywords:** Histidine–rich glycoprotein, Fibrinogen, Zinc ion
IDENTIFICATION OF RESIDUES OF ALPHA-CHAIN OF FIBRIN IMPLICATED IN THE BINDING OF ACTIVATED FXIII A SUBUNIT

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A developing fibrin clot is stabilised and made more resistant to fibrinolysis by the cross–linking abilities of coagulation factor XIII. Independent of cross–linking, thrombin activated FXIII A subunit (FXIIIa) has been shown to bind the alpha–chains of fibrin, between residues 242–424, however the exact residues involved have not previously been determined. To identify these residues the cDNA of alpha–chain 242–424, and truncated variants of this domain, were PCR amplified and cloned into pGEX–6P–1, downstream of a GST fusion tag. The GST alpha–chain fragments were expressed, purified, and the pure alpha–chain fragments isolated by the removal of GST by proteolytic cleavage. Binding interactions between recombinant FXIIIa and alpha–chain variants were analysed using surface plasmon resonance. We can confirm that FXIIIa binds recombinant alpha–chain 242–424 with a KD of 5.2 ± 0.42 microM, and truncated alpha–chain 242–402 (KD 5.64 ± 0.7 microM). However, alpha–chain truncation 242–387 did not bind to FXIIIa. Therefore it has been possible to localise the binding region of FXIIIa to fibrin alpha–chain to within residues 388–424. This work will provide a better understanding of fibrinogen–FXIIIa interactions which could lead to the identification of targets for novel anti–thrombotic therapies.

Keywords: alpha chain fibrin, binding interactions, activated FXIII
The role of fibrinogen in a murine model of lethal endotoxemia

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Sepsis is a condition that results from a host response to infection. Many of the components of the innate immune response that normally facilitate host defenses against infection can, under some circumstances, cause cell and tissue damage and hence multiple organ failure, the clinical hallmark of sepsis. Fibrinogen deposition is a universal feature within injured tissues and inflammatory foci, and appears to locally regulate inflammatory responses. To assess the importance of fibrinogen (Fg) in acute inflammatory damage (LPS), and to determine whether a lack of fibrin deposition will improve the response to the challenge, we have performed studies employing Fg deficient mice. In the present study we show that mice with a total deficiency of fibrinogen present altered responses to a challenge with Lipopolysaccharide (LPS). Survival was increased in these mice and results from histological findings indicate this improvement is correlated with a lack of fibrin deposition in organs and only a minor degree of ischemic damage. Neutrophils appeared early in lungs of challenged wild-type (Wt) mice, while similar infiltration in lungs of mice occurred at later times. This delayed response in Fg deficient mice was confirmed by studies that show a strong dependence on Fg in binding of neutrophils to endothelial cells in the presence of LPS. In plasma, cytokines were elevated as indicators of the inflammatory process; nevertheless in Fg deficient mice the levels were lower at early time points. The time course of MIP-2 expression correlated with the occurrence of pulmonary leakage after LPS challenge, which was delayed in Fg deficient mice. mRNA quantifications of several inflammation– and hemostasis–related gene products indicated that correlations existed between the cytokine levels and mRNA expression. These results suggest that fibrinogen plays a central role in the development of ischemic damage and consequently organ dysfunction, as well as an early mediator in the cross talk between activated coagulation and inflammation.

Keywords: endotoxemia, afibrinogenemia, inflammation
Fibrinogen deficiency and LDL driven atherosclerosis in mice

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Complete deficiencies in mice of both the LDL-receptor (Ldlr−/−) and a key catalytic component of an apolipoprotein B–edosome complex (Apobec1−/−), that converts apoB–100 to apoB–48, has been extensively characterized and applied to an investigation of the role of fibrinogen (Fg) in the genesis and progression of the plaque. Triply deficient mice, Ldlr−/−/Apobec1−/−/Fg−/− (L−/−/A−/−/Fg−/−), presented more advanced plaque in both the aortic trees and aortic sinuses at 24−, 36−, and 48− weeks of age, as compared to L−/−/A−/− mice. The earlier plaque formation in L−/−/A−/−/Fg−/− mice may result from enhanced spontaneous platelet activation in these mice, a feature indicated by the presence of elevated CD61 and CD62P on resting platelets and higher plasma soluble P–selectin in L−/−/A−/−/Fg−/− mice, as compared to L−/−/A−/− and WT mice. The more elevated high molecular weight forms of von Willebrand Factor (vWF), resulting from down-regulation of its cleaving protein ADAMTS13 in L−/−/A−/−/Fg−/− mice, as well as increased vWF collagen binding activity in these same mice, further indicate enhanced activation of their resting platelets, and provides an in vivo mechanism for this effect. Thus, the earlier arterial plaque deposition in L−/−/A−/−/Fg−/− mice appears to contain a contribution from enhanced levels of activated platelets, a consequence of a Fg deficiency, combined with high LDL concentrations.

Keywords: fibrinogen deficiency, atherosclerosis, platelet activation
**FXIII and Thrombin**

**ID:** 167*

**Proteolytic Regulation of Activated Coagulation Factor XIII by Plasmin: A Novel Mechanism to Regulate Thrombus Formation and Clot Stability**

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Factor (F)XIII when activated by thrombin, crosslinks soluble fibrin to increase the resistance of fibrin clots to fibrinolysis. Here we show that activated FXIII (FXIIIa) is preferentially cleaved by plasmin compared to zymogen FXIII, a process that is enhanced in the presence of fibrin (the major physiological substrate of plasmin), and which also occurs in plasma in a time and plasmin dose dependent manner. Degradation of FXIIIa by plasmin occurs prior to fibrinolysis. Clots prepared using plasminogen-depleted plasma do not show degradation of FXIIIa except in the presence of added plasmin. These data identify a novel mechanism for the down-regulation of FXIII activity at the fibrin clot surface. Therefore, plasmin is not only responsible for fibrinolysis directly but also plays a role in limiting the development of a stable thrombus. The results have implications for understanding of normal haemostasis, and the pathophysiology of atherothrombotic cardiovascular- and venous thrombotic-disease.

**Keywords:** FXIII, Regulation, plasmin
IDENTIFICATION OF NOVEL BINDING INTERACTIONS BETWEEN FACTOR XIII AND BOTH TISSUE PLASMINOGEN ACTIVATOR AND PLASMINOGEN

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Factor (F)XIII activity is responsible for increasing the resistance of fibrin clots to fibrinolysis. Plasminogen and tPA both bind to cryptic binding sites on fibrin which are absent on fibrinogen. Consequently, fibrin enhances the rate of plasmin generated by tPA mediated cleavage of plasminogen by 1000-fold. Using surface plasmon resonance, we have identified novel binding interactions between tPA and plasminogen that occur with both zymogen FXIII A2B2 (KD 82.5plus&minus7 nM, ka 1.24plus&minus0.1x10^5 1/Ms, kd 1.02plus&minus0.03x10^-2 1/s and KD 331plus&minus39 nM ka 2.55plus&minus0.3x10^41/Ms, kd 8.36plus&minus0.2x10^-31/s respectively) and thrombin cleaved A2B2 (KD 118plus&minus5 nM, ka 9.47plus&minus0.2x10^4 1/Ms, kd 1.12plus&minus0.02x10^-2 1/s and KD 287plus&minus5 nM ka 2.9plus&minus0.09x10^41/Ms, kd 8.33plus&minus0.1x10^-31/s respectively). These interactions have been confirmed to occur in plasma using immunoprecipitation experiments. The KD of the interaction between plasminogen and zymogen FXIII suggests that in the absence of any other interactions of these proteins, 87% of FXIII will be carried by plasminogen at plasma concentrations. These findings suggest that plasminogen, in addition to fibrinogen, may be involved in the delivery of FXIII to the fibrin clot.

Keywords: Factor XIII, tissue plasminogen activator, plasminogen
SITE-DIRECTED MUTAGENESIS OF RESIDUES SURROUNDING THE THROMBIN CLEAVAGE SITE IN FACTOR XIII-A SUBUNIT DEMONSTRATES RELEVANT SITES FOR INTERACTION

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Background and aims: Factor (F)XIII is a protransglutaminase that on activation by thrombin introduces covalent cross-links in fibrin and incorporates inhibitors of fibrinolysis to increase resistance of the clot to proteolysis. Activation of FXIII involves cleavage of R37-G38 in the A-subunit by thrombin. Interaction with thrombin has been investigated for peptides based on FXIII-A sequence, but not for full-length FXIII-A. The only information on full-length FXIII is derived from the naturally occurring V34L variant, which has been shown to increase activation rates. We therefore used site-directed mutagenesis on recombinant FXIII-A to investigate thrombin interaction.

Methods: Recombinant full-length FXIII-A was expressed with an N-terminal GST-tag in E coli. The cells expressed fully functional FXIII-A of dimeric form. Site-directed mutagenesis was used to produce mutants of the activation peptide and the region downstream of the thrombin cleavage site. Residues 28–35 and 37–42 were mutated to alanine, V29 also to leucine, and V34 to leucine and methionine, to investigate the role of increase of side-chain mass at these positions. Recombinant proteins were purified to homogeneity using glutathione affinity chromatography. Interaction with thrombin was investigated through generation of cross-linking activity in a biotin-pentylamine incorporation assay. Cleavage of the recombinant proteins by thrombin was confirmed by SDS–PAGE.

Results: Mutation of R37 at the thrombin cleavage site to alanine completely abolished activation of FXIII-A by thrombin as expected. Mutation of residues V29, E30, G33 and V34 to alanine significantly reduced activation rates of FXIII-A, indicating a role for these residues of the activation peptide just upstream of the thrombin cleavage site in the interaction between FXIII-A and thrombin. There were no significant effect of mutations of residues G38, V39, N40, L41 or Q42 to alanine on activation rates of FXIII-A, showing that residues immediately downstream of the thrombin cleavage site do not play a role. V34L increased activation rate by thrombin. A similar effect was observed for V29L. Mutation of V34 to different residues showed that an increase in side-chain mass increased activation rates of FXIII-A by thrombin (M>L>V>A34).

Conclusion: These data demonstrate that residues R37, V34 and G33 at P1, P4 and P5 positions play a role in full–length FXIII–A for thrombin interaction. They also indicate a further role for residues V29 and E30 in this process. Side–chain mass at residue 34 enhances thrombin interaction, elucidating a mechanism by which V34L increases FXIII activation rates.

Keywords: Factor XIII, Thrombin, protein interaction
Thrombin upregulates expression of oncostatin M (OSM) in human macrophages and peripheral blood monocytes.

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Background: Hemostatic factors like thrombin play a crucial role in generating thrombotic plugs at sites of vascular damage (atherothrombosis). However, whether hemostatic factors contribute directly or indirectly to the pathogenesis of atherosclerosis remains uncertain. Oncostatin M (OSM) as a member of the IL-6 family of cytokines is a proinflammatory mediator primarily known for its effects on cell growth. We could show recently that OSM induces the expression of plasminogen activator inhibitor–1, an established cardiovascular risk factor, in adipose tissue (Rega et al. Circulation 2005). The aim of the present study was to investigate if thrombin and OSM can act as a link between macrophages, platelets and the development of cardiovascular disease.

Methods: Peripheral blood monocytes (PBMC) were isolated using Ficoll–Paque and CD14 antibodies bound to magnetic MicroBeads®. For macrophage transformation cells were cultivated for 8 – 10 days in the presence of human serum. Plaque macrophages were isolated from atherosclerotic plaques and positive selection of CD14 positive cells was performed employing CD14 antibodies bound to magnetic beads. Cells were incubated with thrombin at different concentrations (0.01 to 1U/ml) for various time periods between 8 and 24 hours. OSM antigen was determined by specific ELISA. OSM specific mRNA was quantitated by RealTime–PCR.

Results: Thrombin increased OSM antigen concentration– and time–dependently up to 20–fold in macrophages and up to 8–fold in PBMC. These results could be confirmed on the mRNA level. In human plaque macrophages stimulation with thrombin resulted in a 5–fold increase of OSM mRNA levels.

Conclusion: Thrombin induces the expression of OSM in human macrophages in vitro. If this effect is also present in vivo it may be a new link between platelets, macrophages and the development of cardiovascular disease.

Keywords: Thrombin, Macrophages, Cardiovascular disease
Gene Regulation

**Transgenic Evidence For The Concentration of t-PA Expressing Sympathetic Nerves in Arterioles**

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**Purpose:** The presence of fine, 0.5–2.0 micron sympathetic nerve fibers embedded in small vessel walls of organs and tissues has long been difficult to visualize in situ. Narrower than most cells, and concealed beneath the seldom-studied adventitia these axon terminals tend to be most plentiful in the walls of 15–100 micron wide precapillary arterioles and nutrient vasa vasora. Recently, they were shown to transport neuron–generated tissue plasminogen activator (t-PA) in vesicles to a few small arteries and arterioles; and to release it abruptly in vitro when stimulated by adrenergic agonists, for which they possess prejunctional receptors. Previous authors have already established that sympathetic neuron analogues– PC 12 and adrenal chromaffin cells–show a similar exocytotic release of t-PA from vesicles via the regulated pathway. But the smallness and inaccessibility of arteriolar axons has hindered efforts to determine whether they are part of a systemic network of t-PA–releasing axons extends to microvascular arterioles throughout the organism. Morphologic confirmation of such a system would i) point to the existence of a large reservoir of neural t-PA within the vast microvasculature, in addition to that in arteriolar endothelium, and ii) further support a larger role for the autonomic nervous system in the regulation of systemic fibrinolysis and proteolysis.

**Method:** To better visualize embedded axons able to express t-PA in situ, we analyzed tissues from a new double transgenic mouse model, to be described, in which expressions of the t-PA promoter and enhanced green fluorescent protein (EGFP) are confined to sympathetic neurons and other neural crest-derived cells.

**Result:** Confocal images to be shown reveal positive expressions most prominently in discrete, varicose axon terminals embedded in the walls of arterioles in the heart, brain, lung, kidney, thymus, spleen and skin; as well as adrenal medulla, bone periostium and marrow. Parent neuron cell bodies, and contiguous sympathetic chain axon bundles show prominent positive expressions. Z axis 3–D images of aortic, carotid, renal and mesenteric artery adventitial layers display much greater numbers of fine co-expressing nerve fibers than earlier immunohistochemical localizations of surface vasa vasora. Uveal melanocytes and retinal ganglion cells (other crest derivatives) also displayed discrete positive localizations.

**Conclusion:** The existence of this t-PA axon network, co-extensive with the peripheral sympathetic nervous system, casts a new light on autonomic–vascular interactions within arterioles. Beyond their prominent endothelial t-PA expression, previously shown, the addition of axon sources identifies arterioles as potential powerhousees of fibrinolysis and plasmin proteolysis within the vast microvasculature. Our hypothesis, to be discussed, is that the arteriolar sympathetic innervation is part of a larger neural crest–derived system for the regulation of plasmin proteolysis during classic ‘fight or flight’ responses.Putative roles of sympathetic t-PA release in arterial proteolytic remodelings, inflammations and lipid depositions, await further study.

**Keywords:** Arteriole, Sympathetic, t-PA

* selected for oral presentation
† not represented at ISFP Congress
Regulation of Urokinase Receptor Expression by Protein Tyrosine Phosphatases

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Urokinase and its receptor uPAR play a major role in several physiological processes such as cell migration, proliferation, morphogenesis and regulation of gene expression. Many of the biological activities of uPA depend on its association with uPAR. uPAR expression and its induction by uPA is regulated at the posttranscriptional level. Inhibition of protein tyrosine phosphatase-mediated dephosphorylation by sodium orthovanadate induces uPAR expression and it was additive with uPA in inducing cell surface uPAR expression. Sodium orthovanadate induces uPAR by increasing uPAR mRNA in a time– and concentration–dependent manner. Both sodium orthovanadate and uPA induce uPAR mRNA stability, indicating that dephosphorylation could contribute to uPA–induced posttranscriptional regulation of uPAR expression. Induction of the tyrosine phosphatase SHP2 in Beas2B and H157 cells inhibited basal cell surface uPAR expression and uPA–induced uPAR expression. Sodium orthovanadate also increased uPAR expression by decreasing the interaction of a uPAR mRNA coding region sequence with phosphoglycerate kinase (PGK) as well as the interaction between a uPAR mRNA 3’untranslated sequence with hnRNPC. On the contrary, overexpression of SHP2 in Beas2B cells increased interaction of PGK with the uPAR mRNA coding region and inhibited hnRNPC binding to the 3’untranslated sequence. These findings confirm a novel mechanism by which uPAR expression of lung airway epithelial cells is regulated at the level of mRNA stability by inhibition of protein tyrosine phosphatase–mediated dephosphorylation of uPAR mRNA binding proteins and demonstrate that the process involves SHP2.

Keywords: Urokinase, urokinase receptor, SHP2
No Evidence for an Association of the Tissue-type Plasminogen Activator –7351C/T Enhancer Polymorphism and the Presence of Stable Coronary Artery Disease or the Occurrence of Acute Myocardial Infarction

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Background. Activation of the coagulation system after rupture or erosion of coronary plaques may lead to acute coronary events. Tissue-type Plasminogen Activator (t-PA) is the major activator of endogenous fibrinolysis that may counteract local thrombus formation. Recently the –7351C/T enhancer polymorphism of t-PA was associated with forearm vascular release of t-PA and with a first myocardial infarction. We tested whether this polymorphism is associated with the presence of stable coronary artery disease (CAD) or the occurrence of acute myocardial infarction.

Methods. We conducted a sex, age and smoking matched case–control study that included 109 patients with acute myocardial infarction, 109 patients with angiographically proven CAD without a history of acute coronary events and 109 controls. Genotyping of the 7351C/T enhancer polymorphism of t-PA was performed by LightCycler.

Results. The prevalence of the t-PA –7351 CC, CT and TT genotypes were 48.6 %, 25.7 % and 25.7 % in controls, 50.9 %, 25.9 %, 23.1 % in patients with stable CAD and in patients with acute myocardial infarction the prevalence of the CC, CT and TT genotypes were 47.7 %, 26.2 % and 26.2 %, respectively. No associations were found between t-PA genotype and myocardial infarction or stable coronary artery disease.

Conclusion. These data suggest that the genetic determination of t-PA plasma levels by the –7351C/T enhancer polymorphism may not play a role in the development of CAD or the onset of acute coronary events.

Keywords: Tissue–type Plasminogen Activa, Polymorphism, Coronary Artery Disease
**Infectious Diseases**

**ID:** 41

**Plasminogen binding on the surface of Leishmania mexicana by enolase**

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Leishmania mexicana is one of the causative agents of cutaneous leishmaniasis on the American continent. L. mexicana has been reported to interact in vitro with plasminogen. This interaction contributes to the virulence of the parasite as may be concluded from differences observed in lesion size and in the distribution pattern in the lesion between plasminogen deficient and wild type mice. In this work we looked for the molecules responsible for plasminogen binding on the surface of the parasite. Using ligand blotting we found that several molecules, with the capacity to bind plasminogen, are found in the microsomal fraction of the parasite. One of these molecules had the same molecular mass of enolase. To test binding of plasminogen to enolase, the purified recombinant enzyme was immobilized on microtitre plates. Plasminogen at different concentrations was incubated and the binding revealed with anti-plasminogen antibodies. A dose-dependent and saturable binding of plasminogen to enolase was observed. The concentration of plasminogen necessary to achieve 50% saturation (EC50) was found to be around 0.3 μM. The binding of plasminogen to control plates coated with BSA was minimal. Binding of plasminogen to immobilized enolase was inhibited up to 80% by the lysine analogue epsilon-aminocaproic acid indicating that the lysine-binding sites of plasminogen are probably involved in this interaction. Immunofluorescence studies with permeabilized and non-permeabilized parasites (promastigotes and amastigote forms) showed that enolase is found at the external face of the plasma membrane. Moreover, plasminogen binding was tested in living parasites in the presence of anti-enolase antibodies. Up to 50% inhibition of plasminogen binding was observed. Thus, enolase could function, besides in metabolism, as plasminogen receptor at the surface of the parasite.

**Keywords:** Leishmania mexicana, plasminogen, enolase
The maintenance of high affinity plasminogen binding by PAM variants from group A streptococci is mediated by both a1 and a2 repeat domains and is not dependent on lysine residues

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Increasingly, subversion of the plasminogen activation system is being implicated in the virulence of Group A streptococci (GAS). GAS display several receptors for human plasminogen on the cell surface including the high affinity plasminogen–binding group A streptococcal M protein (PAM). We have shown that the PAM genotype is associated with Australian Northern Territorial GAS isolates from a variety of disease states and confers high glu–plasminogen binding ability on GAS when compared to PAM–negative GAS isolates. In human plasminogen transgenic mice, a PAM knockout mutant was found to have markedly reduced virulence when compared to the wildtype GAS strain, indicating that plasminogen binding via PAM is an important virulence mechanism for a subset of GAS isolates. The major plasmin(ogen)–binding site of PAM is located in the N–terminal variable region of the protein, and is comprised of two characteristic tandem repeats designated a1 and a2. Internal lysine residues in the a1 and a2 repeat regions (Lys98 and Lys111) are thought to mediate binding to kringle 2. However, binding studies involving a polypeptide designated VEK–30 (comprising the six residues preceding the a1 repeat, the full a1 repeat, together with the first ten residues of the a2 repeat of PAM) and a recombinant kringle 2 modified to contain a high affinity lysine binding site, highlighted a potential role for His102, Arg101 and Glu104 residues within the a1/a2 repeat in this interaction. While the a1 and a2 repeats of PAM are greatly variable, residues Arg101, His102 and Glu104 are highly conserved. It has been suggested that the maintenance of high–affinity plasminogen binding by PAM variants is due to the conservation of these key amino acid residues within the plasminogen binding domain of PAM. In order to further elucidate this novel plasminogen binding interaction, we conducted site–directed mutagenesis studies using full length canonical PAM protein. Mutation of previously identified key residues Lys98, Arg101, His102 and Lys111 within the a1 and a2 repeats reduced, but did not abrogate plasminogen binding by PAM. Loss of plasminogen binding was only observed following simultaneous mutation of Arg101, Arg114, His102 and His115 in both the a1 and a2 repeats regardless of the presence of residues Lys98 and Lys111. To our knowledge, this is the first demonstration of a non–lysine–dependent, high affinity interaction between plasminogen and a full–length naturally occurring receptor. This study also shows that the highly conserved Arg and His residues from either the a1 or a2 repeats in PAM–like proteins mediate high affinity plasminogen binding. This suggests that these residues may compensate for variation elsewhere in the binding repeats, or indeed for loss of one of the repeats, and explain the maintenance of high affinity plasminogen binding by naturally occurring PAM–like variants.

Keywords: group A streptococcus, plasminogen receptor, plasminogen–binding group A st
In situ assays reveal mechanisms by which immunity regulates protective fibrin deposition during infection.

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PURPOSE. Pathways regulating coagulation often become dysregulated during infection, thereby provoking clinically significant pathology. Nevertheless, accumulating data from our laboratory, and others, strongly suggest that infection-associated coagulation has evolved to function protectively. For example, fibrin performs a critical protective function during infection by Toxoplasma gondii, an obligate–intracellular protozoan parasite. In that setting, mice succumb to hemorrhage if they fail to produce fibrin. Remarkably, this hemorrhage is not caused by the parasites themselves but, rather, by the immune system. Thus, fibrin functions protectively during toxoplasmosis by suppressing collateral damage caused by the immune system as it strives to eradicate the intracellular parasites. Here, we investigate mechanisms regulating the deposition of protective fibrin during infection.

METHODS AND RESULTS. Through comparisons of Toxoplasma-infected wild type and cytokine-deficient mice, we dissociate the relative fibrin-regulating capacities of pathogen products, host cytokines, and hemorrhage itself. We demonstrate that neither the pathogen burden nor hemorrhage are primary regulators of fibrin deposition. Rather, cytokines exert dominant and counter-regulatory roles: tumor necrosis factor-alpha and interleukin-6 promote fibrin deposition, while interleukin-10 and interferon-gamma (IFNg) suppress fibrin deposition. To further dissect how immunity regulates infection-associated fibrin deposition we develop assays that quantitatively measure prothrombinase (PTase), protein C-ase (PCase) and plasminogen activator (PA) activities in situ. Using those assays, we demonstrate upregulated PTase, PCase, and PA activity in hepatic tissue of wild type mice infected with Toxoplasma. Despite dramatically upregulating fibrin levels, IFNg-deficiency does not affect the infection-stimulated increase in PTase activity. By contrast, IFNg-deficiency is associated with increased PCase activity and reduced PA activity. Parallel analyses of hepatic gene expression reveal that IFNg-deficiency is also associated with increased expression of thrombomodulin, increased expression of thrombin–activatable fibrinolysis inhibitor (TAFI), and reduced expression of urokinase.

CONCLUSIONS. We conclude that cytokine products of the immune system dictate levels of protective fibrin deposition during infection. We further conclude that IFNg suppresses infection-stimulated hepatic fibrin deposition by reducing thrombomodulin-mediated activation of TAFI, thereby destabilizing fibrin deposits, and concomitantly increasing hepatic urokinase activity, thereby promoting fibrinolysis. Together, our findings reveal specific mechanisms by which the immune system can destroy infected tissue while simultaneously restraining hemorrhage and promoting tissue repair through the deliberate deposition of protective fibrin. This murine toxoplasmosis model permits detailed mechanistic studies of the immunoregulation of infection-associated fibrin deposition and should, thereby, provide novel insights into how infections provoke coagulopathy.

Keywords: infection, fibrin, cytokines
Mice expressing a thrombin variant engineered to favor anticoagulant function are resistant to S. aureus-induced sepsis

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Thrombin has the remarkable attribute that it can act as a prothrombic- or antithrombotic-agent depending on the precise setting. It initiates thrombus formation via PAR–mediated platelet activation and the local conversion of fibrinogen to fibrin, but also negatively-regulates subsequent clot formation though the activation of protein C when bound to thrombomodulin. Intensive structure-function studies over the last decade have revealed that the substrate preferences of thrombin can be “redesigned” to favor either procoagulant or anticoagulant targets by introducing subtle active site alterations. One of the best characterized of these thrombin site-directed mutants is thrombin W215A/E217A (fII–WE), a derivative that strongly favors the antithrombotic substrate protein C over procoagulants. Murine fII–WE has been reported to have markedly reduced activity with fibrinogen (~1200 fold decrease in kcat/Km for fibrinopeptide release) with only modestly reduced activity for protein C activation. The net result is a molecule favoring anticoagulant function. Here, we describe the generation and characterization of mice in which the W215A/E217A mutations have been introduced into the endogenous murine prothrombin gene using a gene-targeting strategy. The mutant allele was transmitted through the germline and was found to support the expression of normal levels of hepatic fII mRNA and plasma fII in both heterozygous and homozygous neonates. A typical Mendelian distribution of fII–WT/WT, fII–WE/WT and fII–WE/WE was observed in E18.5 embryos derived from heterozygous breeding pairs, but a fraction of homozygous mutants displayed hemorrhagic pathologies in utero. Consistent with a profound defect in procoagulant function, homozygous fII–WE/WE offspring uniformly succumbed to spontaneous bleeding events within days of birth. Heterozygous fII–WE/WT animals generally survived to adulthood and were capable of carrying multiple liters to term, but consistent with a predicted dominant–negative phenotype about 5% of fII– WT/WE neonates developed fatal perinatal bleeding events and adults exhibited extended bleeding times following tail tip excision. In surviving fII–WE/WT adults, complete blood cell counts and standard PT and aPTT clotting times were indistinguishable from wildtype littermates. Given that activated protein C has been shown to be efficacious in the treatment of sepsis, we explored whether the shift in thrombin substrate specificity in heterozygous fII–WE/WT mice would have the benefit of rendering animals tolerant to acute septic challenges relative to wildtype mice. Kaplan–Meier analysis following intravenous administration of S. aureus revealed a significant survival advantage for fII–WE/WT mice relative to littermate controls over a 7 day observation period. In addition to providing a valuable new resource for further understanding the role of thrombin in hemostasis and the host inflammatory response, fII–WE mice illustrate the therapeutic potential of recombinant prothrombin derivatives with redesigned specificity.

Keywords: thrombin, gene–targeting, infection
ORGAN-SPECIFIC EXPRESSION OF THE PLASMINOGEN ACTIVATING SYSTEM IN A BABOON MODEL OF SEPSIS

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Background: Sepsis is an excessive systemic inflammatory response to infection that is associated with an increased procoagulant state leading to disseminated intravascular coagulation. Fibrin formation is counteracted by activation of the plasminogen activating (PA) system, responsible for the plasmin–dependent extracellular proteolysis. PA system comprises activators [tissue–type PA (t–PA) and urokinase–type PA (u–PA)], inhibitors [PA inhibitors 1 and 2 (PAI–1, PAI–2)], and cellular receptors, u–PA receptor (u–PAR). While the plasma values of the PA proteins during sepsis are well characterized, tissue and organ– specific expression is largely unknown. Aim: to characterize the organ–specific expression of the PA system proteins in five vital organs – lung, kidney, heart, liver, spleen– in baboons challenged with E. coli, as compared to healthy animals. Experimental Procedure and Methods: Three animals/group were intravenously injected with sublethal doses of E. coli (SLEC; 10x9 CFU) and sacrificed after 2, 8, 24 hours, or with a lethal dose (LDEC; 10x10 CFU) and sacrificed at the time of death. Non-challenged animals served as controls. Gene expression analysis involved semi–quantitative RT–PCR. Protein extracts were analyzed by Western blot and ELISA. Organ–specific localization of the PA proteins and the degree of fibrin deposition in the investigated organs were visualized by confocal microscopy.

Results: t–PA mRNA and protein levels were gradually decreased in the heart and kidney, but were up–regulated in the lung, liver, and spleen of the SLEC challenged baboons vs. controls. In LDEC baboons, t–PA mRNA and proteins were strongly increased in all organs (up to 4 fold) except for the heart, where it was about 4 fold decreased vs. controls. u–PA levels were moderately increased in the heart, liver and spleen and were steadily decrease in kidney, which also, showed the highest amount of fibrin deposition. PAI–1 mRNA and protein were gradually and constantly increased in all analyzed organs, which suggests an important role in sustaining the procoagulant state in sepsis. PAI–2 mRNA shown increased levels only in the spleen of SLEC treated baboons, while PAI–2 protein was strongly increased in most of the organs, especially in LDEC challenged animals. Both PAI–1 and PAI–2 tissue and plasma levels were consistently associated with lethality in baboons. Western blot analysis showed organ heterogeneity in PA proteolytic potency: while in the lung most of uPA was in complexes with PAIs, in the liver it was mainly in the free form. In SLEC treated animals, uPAR mRNA was slightly upregulated in kidney, lung and liver and steadily downregulated in heart. This pattern was also mirrored in the LDEC treated animals.

Conclusion: Disregulated fibrinolysis in blood and tissue of septic baboons positively correlated with widespread fibrin deposition, which may contribute to multiple organ failure. A thorough understanding of the tissue expression of the PA proteins will enhance our understanding of the pathophysiological mechanisms underlying the sepsis–associated coagulopathy and is a prerequisite for a rational approach and future therapy of severe sepsis.

Keywords: sepsis, inflammation, plasminogen activators
von Willebrand Factor Binding Protein is a Novel Conformational Activator of Prothrombin that Functions Through a Substrate-assisted Molecular Sexuality Mechanism

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von Willebrand factor binding protein (vWbp), a member of the zymogen activator and adhesion protein (ZAAP) family, is composed of NH2-terminal staphylocoagulase–homologous D1 and D2 domains and a COOH-terminal region that adheres to von Willebrand factor (vWf). The prototypical ZAAP is staphylocoagulase (SC); a Staphylococcus aureus secreted protein that activates ProT non–proteolytically by the “molecular sexuality” mechanism, in which insertion of the SC NH2–terminus into the NH2–terminal binding cleft on the ProT zymogen induces conformational activation of the catalytic domain. Based on the predicted SC–like fold of the D1–D2 homologous fragment, vWbp–(1–262), this fragment and full–length vWbp–(1–474) were cloned from the vancomycin–resistant human pathogen Staphylococcus aureus Mu50. Conformational activation of ProT in the presence of vWbp–(1–262) or vWbp–(1–474) was demonstrated by covalent labeling of the ProT catalytic site with a fluorescence probe attached by a thioester peptide chloromethyl ketone inhibitor. These results indicated that the NH2–terminal SC–homologous D1–D2 portion of vWbp was sufficient to activate ProT non–proteolytically. To determine whether vWbp functions via the molecular sexuality mechanism, NH2–terminal mutants vWbp–(2–474) and vWbp–(3–474) were assessed for their ability to activate ProT. Chromogenic substrate measurements of ProT activation indicated that vWbp–(2–474) had approximately 75% reduced ability to activate ProT and vWbp–(3–474) had no detectable activity. Enzyme kinetics analysis of ProT activation by vWbp–(1–262) using a thrombin–specific chromogenic substrate (H–D–Phe–Pip–Arg–pNA) showed a time–dependent increase in the rate of substrate hydrolysis that was enhanced by increasing activator and substrate concentration. The results supported a hysteretic model of ProT activation, in which the initially formed, inactive vWbp–(1–262)–ProT complex is in an unfavorable equilibrium with the active form that is shifted toward the active complex by binding of the chromogenic substrate. Equilibrium binding studies by tryptophan fluorescence showed a ProT fluorescence enhancement upon addition of vWbp–(1–474), consistent with vWbp binding to a single site with a dissociation constant of 31 nM. Preliminary comparisons of dissociation constants obtained for vWbp–(1–262) from kinetic studies and that obtained for vWbp–(1–474) from equilibrium binding suggest that full–length vWbp binds ProT with approximately 26–fold higher affinity compared to vWbp–(1–262), and about 430–fold lower affinity than SC–(1–325). Results of these studies support the conclusion that vWbp activates ProT conformationally through the molecular sexuality mechanism. The vWbp mechanism appears distinct from that of SC in that the rate of vWbp–mediated activation may rely on the presence of an appropriate physiological substrate. This novel conformational ProT activator may utilize distal COOH–terminal interactions to localize ProT activation and fibrin deposition to vWf–rich sites during acute staphylococcal endocarditis. Supported by NIH Grant HL071544

Keywords: prothrombin, endocarditis, von Willebrand factor
Minor Edema Formation after DSPA-induced Clot Lysis of Experimental Intracerebral Hemorrhages

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Purpose of the study: Since Desmoteplase (DSPA) differs from recombinant tissue plasminogen activator (rtPA) in its higher fibrinogen affinity and absence of excitotoxicity, we regarded it as a key candidate for local fibrinolysis of experimental intracerebral hemorrhages.

Methods used: In 17 pigs with a body weight of 30–35 kg, a balloon was inflated in the right frontal white matter to create a preformed cavity. Subsequently, autologous venous blood was injected ICP-controlled via a 1.2 cm-long catheter attached to a subgaleal Rickham reservoir. After MR imaging, DSPA (n=7) or rtPA (n=4) were injected in equimolar concentrations into the hematoma immediately after hematoma induction. In 6 control pigs, the hematoma was not treated at all. For hematoma and edema quantitation, MR imaging was repeated using T2* weighted and FLAIR sequences 4 and 10 days after hematoma induction.

Summary of the results: In the DSPA-treated pigs, the hematoma significantly reduced from 1.36±0.45 cm³ to 0.43±0.37 cm³ (p<0.02) and the increase in edema size from 0.92±0.78 cm³ to 1.01±1.7 cm³ within 10 days was not significant as opposed to both the rtPA-treated and control pigs. The inflammatory reactions measured on EvG-stained slices was significantly less in DSPA-treated pigs compared with rtPA-treated pigs.

Conclusion reached: Local administration of DSPA exerts a minor degree of edema formation and inflammatory reactions in pigs with intracerebral hemorrhages. For its lacking excitotoxic properties, DSPA appears favourable to rtPA in local clot lysis.

Keywords: Desmoteplase, intracerebral hemorrhage, brain edema
The Role of Endogenous versus Exogenous tPA on Edema Formation in Murine ICH

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Purpose of the study: To minimize the neurotoxic injury by clot–derived substances after intracerebral hemorrhage (ICH) on the surrounding brain tissue, minimally-invasive neurosurgical protocols have evolved evacuating the hematoma by stereotaxic injection of a fibrinolytic agent such as recombinant tissue plasminogen activator (rtPA), followed by aspiration of the lysed clot. However, the possible contribution of the presence of exogenous tPA itself to the toxic effects of hematoma-derived factors complicates the rationale for and efficacy of this therapeutic approach. To clarify the role of exogenous rtPA on edema development, we examined the extent of edema formation in a murine model of collagenase-induced ICH, which included tPA-deficient (tPA−/−) and wild-type (wt) mice.

Methods used: In 16 (7 tPA−/− and 9 wt mice) out of 32 mice, 1 mg/kg rtPA was injected into the hematoma 5 hours after ICH induction followed by aspiration of the liquefied clot 20 minutes later. In the control group (8 tPA−/− and 8 wt mice), only collagenase was injected. The edema volume was quantified using SPOT software on Luxol Fast Blue and Cresyl violet-stained cross sections 24 hours, 3, and 7 days post surgery.

Summary of the results: 24 hours after ICH induction, tPA−/− mice had a significantly smaller edema volume (p<0.01), even when rtPA was administered. Between days 3 and 7 after ICH, exogenous rtPA exerts its edema promoting effect irrespective of the underlying genotype and exhibits an extensive microglial activation adjacent to the clot.

Conclusion reached: The role of the endogenous tPA appears to be limited to the early phase of edema formation, whereas exogenous rtPA is edema-promoting between days 3 and 7 after ICH.

Keywords: intracerebral hemorrhage, tissue plasminogen activator, microglia
Elevation of complement C3 and C-reactive protein in men with myocardial infarction

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Atheroma formation, plaque rupture and thrombosis represent the pathophysiological processes underlying the development of acute coronary syndromes (ACS). Evidence indicates a fundamental role of inflammatory processes in all stages of development of ACS. Aim. The aim of this study was to determine the associations of plasma complement C3 and C-reactive protein with MI in a case–control study. Methods. 342 Caucasian male subjects aged ≤ 65 years with acute MI diagnosed according to WHO criteria, and 197 control subjects matched for age, gender, race and domicile were recruited from the Leeds General Infirmary, Leeds, UK. Fasting blood samples were taken at least 2 months after the acute event to minimise the bias of an acute phase response. Plasma CRP and C3 levels were determined by ELISA. Statistical analyses were carried out using SPSS v12, and data presented as mean (95% confidence intervals). Results. Complement C3 was significantly higher in patients with MI (1.19 [1.16, 1.22] g/L) compared with controls (0.99 [0.94, 1.03] g/L, p=0.001). CRP was also significantly higher in patients (1.41 [1.24, 1.60] mg/L) compared with controls (0.72 [0.61, 0.85], p=0.001). In a logistic regression model including C3, age, smoking, diabetes and hypertension, C3 was independently associated with MI; the odds ratios (ORs) for MI in subjects with C3 in quartiles 2, 3 and 4 compared to those with C3 in quartile 1 were: 2.65 [1.56, 4.50], 4.12 [2.36, 7.22] and 7.88 [4.20, 14.77] respectively. CRP was also independently associated with MI in a model including age, smoking diabetes and hypertension; the ORs for those with CRP in the 2nd, 3rd and 4th quartiles compared to the 1st were: 1.35 [0.80, 2.27], 2.01 [1.17, 3.45] and 2.49 [1.40, 4.42], respectively. To evaluate the combined effect of elevated C3 and CRP, subjects were classified into 4 groups according to whether C3 and CRP levels were above or below the median (1.12 g/L and 1.08 mg/L, respectively). 49% of controls and 21% of patients had C3 and CRP levels below the median, 22% of controls and 18% of patients had only CRP levels above the median, 15% of controls and 21% of patients had only C3 levels above the median and 14% of controls and 41% of patients had both CRP and C3 above the median. In a logistic regression model including age, smoking, diabetes and hypertension, compared to subjects with both C3 and CRP below the median, the odds ratio for MI in subjects with elevated CRP alone was 1.58 (0.92, 2.70), p=0.095, for elevated C3 alone was 3.06 (1.74, 5.40), p<0.001 and for elevated C3 and CRP was 4.79 (2.77, 8.28), p<0.001. Conclusion: These data suggest that elevated C3 is independently associated with MI and that evaluation of C3 in addition to CRP may be more beneficial than either inflammatory variable alone in assessing risk for MI.

Keywords: complement C3, C-reactive protein, myocardial infarction
Distinct Engagement of Integrin alphaMbeta2 by Plasminogen and Angiostatin Controls Neutrophil Apoptosis.

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Neutrophil (PMN) accumulation in the inflamed tissue is determined by the balance between the rate of their recruitment and removal, which must be tightly regulated to maximize host defense and limit destructive effects of PMN cytotoxicity. PMN recruitment requires cooperation between chemokines, leukocyte adhesion molecules and extracellular proteases. On the other hand, PMN clearance from tissues is achieved by their apoptosis and is crucial for the successful resolution of inflammation. We recently reported that integrin alphaMbeta2 can mediate pericellular proteolysis and directly interacts with plasminogen (Plg) and its activator urokinase (uPA) to significantly accelerate plasmin (Plm) generation, events which facilitate PMN invasion through extracellular matrices. In the present study, we have sought to determine whether alphaMbeta2 interaction with Plg affects spontaneous apoptosis of PMN, a process controlled by the beta2 integrins. As has been assessed by Anexin V binding, when human PMNs were incubated with soluble Plg for 16h, only 14% of cells were apoptotic in contrast to 59% of untreated PMNs. Angiostatin [Ang(1-4)], containing the first 4 kringle domains of Plg, bound to alphaMbeta2 but was not protective. The Plg-mediated PMN survival was dependent upon engagement of alphaMbeta2 as it was blocked by NIF (Neutrophil Inhibitory Factor), a high affinity ligand of the integrin, which also inhibits Plg binding to alphaMbeta2. The observed apoptosis involved activation of a pro-apoptotic factor bax and caspases 3 and 8, and Plg-promoted PMN survival was associated with inhibition of their cleavage. In addition, alphaMbeta2 engagement with Plg, but not with Ang(1-4), induced phosphorylation of Akt and ERK1/2 kinases, which were pivotal for PMN survival as specific inhibitors of these kinases abrogated the effect of Plg on PMN survival. To delineate the mechanism of distinct effects of Plg and Ang(1-4) on cell survival we transfected K562 cells with entire alphaMbeta2 heterodimer, with separate alphaM or beta2 integrin subunits or with an empty vector. Plg promoted survival of alphaMbeta2–transfected K562 cells, in contrast to the cells expressing single alphaM or beta2 subunit and mock cells, which were not protected by Plg. Ang(1-4) failed to prevent apoptosis in all tested cell lines. In addition, Plg induced activation of Akt and inhibited cleavage of bax and caspases only in alphaMbeta2–transfected cells, while not in alphaM–, beta2– or mock cells. When interactions of both ligands with K562 cell lines were tested in cell adhesion and soluble ligand binding assays, Plg was recognized by the alphaMbeta2, alphaM– and beta2– cells, but not by mock–transfected cells, and Ang(1-4) bound to the alphaMbeta2– and alphaM–cells, while it did not interact with the beta2– and mock cells. Finally, Plg prevents leukocyte apoptosis via engagement of both subunits of alphaMbeta2, while Ang(1-4) fails to do so as it does not interact with the entire heterodimer, but only with the alphaM subunit. Thus engagement of alphaMbeta2 with Plg not only focuses proteolytic activity to PMN surface facilitating PMN migration but also induces intracellular signaling events that protect these cells from spontaneous apoptosis, supporting PMN accumulation in a site of inflammation.

Keywords: neutrophil apoptosis, plasminogen, integrin
Membrane Proteases

**ID:** 174*

**Matriptase is the essential target of hepatocyte growth factor activator inhibitor-1 during embryonic development**

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Hepatocyte growth factor activator inhibitor-1 (HAI-1) is a Kunitz-type transmembrane serine protease inhibitor that forms inhibitor complexes with several trypsin–like serine proteases and is required for mouse development. Here we show that the single essential function of HAI-1 in embryonic development is to restrict the activity of the type II transmembrane serine protease, matriptase, which is itself dispensable for development. Enzymatic gene trapping of matriptase combined with HAI-1 immunohistochemistry revealed that matriptase was coexpressed with HAI-1 in both extraembryonic and embryonic tissues. As early as embryonic day 8.5, matriptase and HAI-1 were expressed in a small population of undifferentiated trophoblasts of the chorion. In the absence of HAI-1, the integrity of this population of matriptase expressing chorionic trophoblasts was perturbed, as evidenced by basement membrane dissolution, altered E–cadherin and beta–catenin protein expression and localization, and aberrant morphology. This prevented chorionic trophoblast invasion of the spongial layer and formation of the placental labyrinth. Matriptase ablation in HAI-1-deficient embryos restored the integrity of chorionic trophoblasts and enabled both placental labyrinth formation and development to term. Furthermore, matriptase/HAI-1 double-deficient mice were phenotypically indistinguishable from matriptase single-deficient littermate controls. These data show that matriptase is the essential target for HAI-1 during mouse embryonic development and provide the mechanistic basis for the failure of placental formation in HAI-1-deficient mice.

**Keywords:** membrane proteolysis, trophoblast invasion, placentation
**BIOCHEMICAL CHARACTERIZATION OF PROSTASIN, THE EPITHELIAL SODIUM CHANNEL ACTIVATING PROTEASE**

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Human prostasin was recently identified as a potential activator of the epithelial sodium channel (ENaC). We report here the biochemical characterization of prostasin activity and substrate preferences in vitro. Positional scanning combinatorial substrate libraries were used to demonstrate a preference by prostasin for poly-basic substrates: in position P4 preference was for arginine or lysine; in P3 preference was for histidine, lysine or arginine; in P2 preference was for basic or large hydrophobic amino acids; and in P1 preference was for arginine and lysine. P1’, P2’, and P3’ displayed broad selectivity with the exception of a lack of activity for isoleucine. P1’ had a preference for small, unbranched, amino acids. Using these substrates, prostasin activity was found to be highly influenced by metal ions, which were potent inhibitors and substrate specific modulators of enzymatic activity. In the presence of sub-inhibitory concentrations of zinc, the activity of prostasin increased several-fold and its substrate specificity was significantly altered. In addition, the substrate preferences provide insight into prostasin regulation and signal transduction. For example, the absence of activity seen with substrates containing isoleucine in position P1’ explains the inability of prostasin to autoactivate and suggests that prostasin proteolytic activity is regulated by an upstream protease. Furthermore, a potential prostasin-cleavage site was found in the extracellular domains of the ENaC alpha- and gamma-subunits, and may present a mechanism for direct ENaC activation by prostasin.

**Keywords:** sodium, processing, proteomics
Tetraspanin associations with membrane type-1 matrix metalloproteinase (MT1-MMP)

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Tetraspanins are a family of cell surface proteins that possess 4 transmembrane domains. They function as organizers of multimolecular membrane complexes, thereby regulating a variety of cell functions such as cell migration, fusion and signaling events. Membrane type-1 matrix metalloproteinase (MT1-MMP) is a membrane anchored member of the matrix metalloproteinase (MMP) family of extracellular proteolytic enzymes. MT1-MMP has the capacity to remodel the extracellular matrix and to shed a variety of transmembrane proteins from the cell surface. Thus far there has been little evidence linking tetraspanins with MMPs including MT1-MMP. During the course of our studies of tetraspanins, we noticed that several members of the tetraspanin family can be selectively co-immunoprecipitated with MT1-MMP. Tetraspanin–MT1-MMP interactions are especially obvious under mild detergent conditions from a number of cell lines. These interactions appear to require the hemopexin domain of MT1-MMP together with additional sequences from the MT1-MMP transmembrane domain. Consequently, mutant MT1–MMP proteins lacking these domains are impaired in their MT1–MMP–tetraspanin associations. The cytoplasmic tail, catalytic domain and pro–domain of MT1–MMP are dispensable for associations with tetraspanins. We hypothesize that tetraspanin association with MT1–MMP may affect MT1–MMP functionality and subcellular localization. Studies are underway to test this hypothesis.

Keywords: tetraspanin, matrix metalloproteinases (MMP, membrane type–1 matrix metallo
**MMP’s and TIMPS**

**ID: 4* **

**Inhibitory Effects of Green Tea Catechins and Lignans on the Activity of Human Matrix Metalloproteinase 7 (MMP-7) and Insights into Their Structure-Activity Relationship**

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MMPs are considered to be involved in the tumor metastasis and invasion, and potent and safe MMP inhibitors have been desired for tumor therapy. The inhibitors could be also useful tool to regulate the enzyme activity and to explore the enzyme reaction mechanism. In this study, inhibitory effect of 10 catechins and 9 dibenzylbutyrolactone lignans on MMP-7 activity was examined in the hydrolysis of a synthetic substrate, MOCac–PLGL(Dpa)AR, in 50 mM HEPES buffer (pH 7.5) containing 10 mM CaCl2. The catechins examined were classified into three groups according to their inhibition potency: (+) and (-)-catechins, and (+) and (-)-epicatechins (EC) had Ki of > 1 mM; (-)-gallocatechin (GC) and (-)-epigallocatechin (EGC) had Ki of > 0.05 mM; and catechins with a galloyl group at the 3 position [(+)-catechin–3–gallate (CG), (+)-epicatechin–3–gallate (ECG), (+)-gallocatechin–3–gallate (GCG), and (+)-epigallocatechin–3–gallate (EGCG)] had Ki values of 400–1600 nM. The inhibitory potency of ECG was the strongest among them. A major component of green tea catechin, EGCG, is in the third group. The inhibition manner of the catechins in the third group against MMP-7 was shown to be non-competitive. The potency of EGCG was not affected by the presence of an inhibitor ZnCl2, suggesting that the inhibitions of MMP-7 by EGCG and by ZnCl2 might be independent of each other. The inhibitory effects of green tea catechins suggest that a high intake of green tea might be effective for the prevention of tumor metastasis and invasion in which MMP-7 is concerned. All of the lignans examined inhibited MMP-7 with the IC50 ranging from 0.05 to >0.28 mM. Mataitresinol, which has the basic structure of the other lignans, showed the weakest inhibition. Lignans with methylenedioxy ring(s) or a hydroxyl group at the C5–position inhibited MMP–7 more strongly than mataitresinol. 5–Hydroxypluviatolide, which has both a methylenedioxy ring and a hydroxyl group at the C5–position, was the most potent inhibitor (IC50 = 0.05 mM), suggesting that the introduction of these two elements might enhance synergistically the inhibitory activity of lignans. 5–Hydroxypluviatolide inhibited MMP–7 in a competitive manner, and its inhibitory effect was greatly suppressed by the presence of another competitive inhibitor, dimethyl sulfoxide. The precursors of mataitresinol, coniferol alcohol and secoisolariciresinol, had no inhibitory activity, indicating that the dibenzylbutyrolactone structure is essential for the inhibition. Lignans were shown to have the potential to inhibit MMP–7, and the knowledge of their structure–function relationship might be beneficial to developing selective inhibitors for MMPs. It should be noted that the inhibitory manner of catechins was non–competitive, whereas that of lignans was competitive. This suggests that there must be an activity–regulatory site, which accommodates catechins specifically but not lignans, other than the active site which accommodates lignans specifically but not catechins. Catechins and lignans must be suitable proves for exploring the regulatory site as well as the active site of MMP–7 and could be lead compounds for developing inhibitory drugs against MMP–7.

**Keywords:** MMP–7, inhibitor, catechins and lignans
The induction of matrix metalloproteinases by endotoxin in vivo is modulated by short term simvastatin-treatment

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Background. Situations associated with endotoxemia like surgery or sepsis are associated with an increased cardiovascular morbidity and mortality. Matrix metalloproteinases (MMPs) may lead to coronary plaque destabilization by degradation of extracellular matrix. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors have been shown to down-regulate MMPs in vitro. In this study, we examined the role of statins in experimental endotoxemia on the expression of MMPs and their inhibitor tissue inhibitor of metalloproteinases-1 (TIMP-1) in vivo.

Methods and Results. In this randomized, double-blind, placebo controlled study, 20 healthy male volunteers (mean age 27±2) were randomized to receive either simvastatin 80 mg daily or placebo for 4 days. On day four, LPS (20 IU/kg) was administered intravenously and serum samples were taken at baseline and 4 and 8 hours after LPS administration. LPS significantly up-regulated serum levels of MMP-3 (p<0.05) and MMP-9 (p<0.001). In contrast, serum levels of MMP-1 and TIMP-1 were not affected by LPS administration. Whereas simvastatin-treatment had no effect on MMP-9 serum levels, the LPS-induced up-regulation of MMP-3 was suppressed by statin administration (p<0.01).

Conclusions. Short term, high dose simvastatin treatment blunts the response of MMP-3 to endotoxemia in vivo. Statins may thereby have a protective effect on the cardiovascular system during surgery or sepsis.

Keywords: matrix metalloproteinases, statin, endotoxin
HIGH MMP-9/NGAL COMPLEX LEVELS IN GASTRIC CANCER TISSUE ARE ASSOCIATED WITH WORSE SURVIVAL

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Matrix metalloproteinases (MMPs) are involved in tissue remodelling, tumour invasion and metastasis. High levels of gelatinases MMP-2 and MMP-9 in various types of cancer tissue are associated with worse survival of the patients. Complexes of MMP-9 with neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin-2) were found in urine from breast cancer patients but were absent in healthy controls, suggesting a possible application as tumour marker. We analyzed the presence of MMP-9/NGAL complexes in tissue from gastric cancer patients and determined their possible clinical value. MMP-9, NGAL, and MMP-9/NGAL complexes were determined in 81 tissue homogenates from gastric cancer patients using quantitative zymography, ELISAs and a specific MMP-9 bioactivity assay. The tumour levels were examined for associations with established clinico-pathological parameters including classifications according to TNM, WHO, Laurén, and survival. Gastric carcinomas were found to have significantly increased MMP-9 (P<0.001), NGAL (P=0.002), and MMP-9/NGAL (P<0.001) levels compared to normal gastric mucosa. Immunohistochemistry revealed staining for MMP-9 mainly in neutrophils, occasionally in epithelial cells and endothelial cells, and incidentally in muscle cells, macrophages, and fibroblasts. NGAL was similarly distributed in neutrophils and epithelial cells, but was also present in groups of epithelial cells in which no MMP-9 was detected. Endothelial cells and fibroblasts showed little or no staining for NGAL. In the homogenates, NGAL was more abundantly present than MMP-9, in specific cases more than 100 times higher. Active MMP-9 levels correlated significantly with the total antigen level of MMP-9, but also with MMP-9/NGAL levels, confirming a possible protective role for NGAL-complex formation in MMP-9 (auto)activation. Although all gastric tumour subtypes showed higher MMP-9/NGAL levels than their corresponding normal tissue, only classification of the carcinomas according to Laurén and WHO showed significant differences between tumour subtypes. High levels of MMP-9/NGAL complexes in gastric tumours were significantly associated with worse survival in Cox’s univariate (HR 2.087, P=0.006) and multivariate analysis (HR 2.095, P=0.025), whereas the levels of NGAL and MMP-9 alone were not indicative for survival. Conclusions: MMP-9/NGAL complexes are significantly enhanced in gastric carcinomas and are significantly associated with worse survival of the patients. The prognostic value of the complexes is in accordance with the presumed role of NGAL, i.e. the protection against autoproteolytic activity of MMP-9 after it has been released from the cells.

Keywords: MMP-9, lipocalin, prognosis

* selected for oral presentation
Enhanced antigen levels of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are associated with clinico-pathological parameters of the tumors and survival of patients with gastric cancer. Recently, Single Nucleotide Polymorphisms (SNPs) in MMP- and TIMP-genes have been associated with susceptibility for various diseases. In this study, we studied the genotype distribution and allele frequencies of SNPs of MMP-2, -7, -8 and -9 and TIMP-1 and -2 in gastric cancer patients in relation to tumor progression, patient survival, and tissue antigen expression. Genomic DNA was isolated from tissue of 81 Caucasian gastric cancer patients and from blood of 169 controls. Genotypes were analyzed by PCR based techniques. Antigen levels for MMPs and TIMPs were determined in tissue homogenates from the same patients using specific ELISAs. Genotype distribution and allele frequencies of MMP-2, -7, -8, -9 and TIMP-1 and -2 were similar in gastric cancer patients and controls, except for MMP-7-181 A>G. In addition, the genotype distribution of MMP-7-181 A>G was associated with H. pylori status (X² 7.8, P=0.005) and tumor-related survival of the patients (Log Rank 3.57, P=0.059). SNP TIMP-2303C>T correlated significantly with the WHO classification (X² 5.9, P=0.03) and strongly with tumor-related survival (Log Rank 11.74, P=0.0006). SNPs of MMP-2, -8, -9 and TIMP-1 were not associated with tumor-related survival. Only the MMP-2-1306 C>T polymorphism, located in the promoter of the gene, correlated significantly with the protein level within the tumors. First order dendrogram cluster analysis combined with Cox analysis identified the MMP-7-181 A>G and TIMP-2303C>T polymorphism combination to have a major impact on patients survival outcome.

Conclusions: Determination of MMP-related SNPs, especially MMP-7-181 A>G and TIMP-2303C>T, might be a useful tool to stratify and select patients for primary resection and (neo)-adjuvant treatment of gastric cancer aiming at better outcome. In addition, upregulated protein level of MMP-2, associated with a promoter-located SNP, is a consistent independent prognostic factor in gastric cancer.

Keywords: MMP, SNP, survival
INFLIXIMAB INDUCES A GENOTYPE-DEPENDENT MUCOSA PROTECTIVE MATRIX METALLOPROTEINASE PHENOTYPE IN INFLAMMATORY BOWEL DISEASE

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Previous studies have shown an upregulation of Matrix Metalloproteinases (MMPs) in intestinal tissue of patients with inflammatory bowel disease (IBD) and a significant clinical and endoscopical improvement of the patients after administration of the anti-TNF-α antibody Infliximab. We determined ex vivo the expression and secretion of MMP-1, -2, -3, -9 and their inhibitors TIMP-1, -2 by IBD affected mucosa vs control intestinal mucosa, to assess the regulatory capacity of Infliximab on the proteolytic phenotype. Intestinal mucosal explants from 20 IBD and 15 control patients, were cultured in medium with or without Infliximab and/or the T-cell activator pokeweed mitogen (PWM). Explants and culture supernatants were analysed for MMPs, TIMPs and TNF-α protein, activity and/or mRNA levels by respectively ELISA, BIA and semi-quantitative RT-PCR. All patients were genotyped for functional TNF-α, MMP and TIMP single nucleotide polymorphism (SNP) loci by RFLP or tetra primer ARMS PCR. The expression of MMP and TIMP protein/activity in basal medium was higher in IBD vs control explants. Infliximab downregulated genotype-dependently MMP-1, MMP-3 and MMP-9 relative to TIMP-1, -2, and also decreased MMP-1 and MMP-3 activities, while PWM enhanced these levels, partly counteracted again by Infliximab. The expression of MMP-2 relative to TIMP did not change by treatment with Infliximab and/or PWM. Conclusions: The high expression of MMPs in patients with IBD suggests a role for these proteinases in the pathogenesis of the disease. Infliximab induces a genotype-associated matrix protective phenotype by down regulating MMP-1, -3 and -9 relative to TIMP, which may contribute to the observed therapeutic efficacy of this drug in IBD, particularly at the mucosal surface.

Keywords: TNF-α, inflammation, MMP
Terminal sialylation regulates autolysis of cell surface-associated membrane type-1 matrix metalloproteinase (MT1-MMP)

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Invasion-promoting membrane type-1 matrix metalloproteinase (MT1–MMP) functions in cancer cells as an oncogene and as a mediator of proteolytic events on cell surface. The net activity of cell surface–associated MT1–MMP is post-translationally regulated through several processes including the processing of the proenzyme by furin–like proprotein convertases, inhibition by the tissue inhibitor of MMPs (TIMPs), autolytic degradation, shedding, exocytosis, endocytosis, and recycling. Here, we demonstrate that, in addition to these already known mechanisms, MT1–MMP is also regulated by O–glycosylation of its proline–rich hinge domain. Insignificant autolytic degradation is characteristic of endogenously expressed MT1–MMP. In turn, extensive autolytic degradation, which leads to the inactivation of the proteinase and the generation of its C–terminal membrane–tethered degraded species commencing at Gly285, is a feature of overexpressed MT1–MMP. Earlier work experimentally demonstrated that MT1–MMP is post–translationally modified by O–glycosylation of the Thr291 – Ser301 hinge region sequence. In the peptide sequence of MT1–MMP, these sites are adjacent to the autolytic cleavage site at Gly285. On the basis of these data, we hypothesize that glycosylation contributes to the stability of cellular MT1–MMP and that “incomplete” glycosylation promotes autolytic degradation of the recombinant, overexpressed, MT1–MMP enzyme. Our experimental results are consistent with this hypothesis and also with earlier data presented by other laboratories. In the course of our study, we initially confirmed that overexpressed MT1–MMP was significantly degraded in many cell systems, including breast carcinoma, colon carcinoma, glioma, fibrosarcoma, Madin–Darby canine kidney, embryonic kidney and Chinese hamster ovary cells. We next determined that BGN, a competitive inhibitor of O–glycosylation, significantly promoted the autocatalytic degradation of MT1–MMP. By cell surface biotinylation experiments using a cleavable biotin reagent, we also demonstrated that glycosylation affects the uptake rate of MT1–MMP forms. We also identified a previously undetected glycosylated intermediate of MT1–MMP that resided predominantly in the intracellular compartment and that was inefficiently transported to the plasma membrane. On the other hand, in the course of glycosylation, sialic acid frequently terminates the carbohydrate moiety. Our results also indicated that the enzymatic removal of the terminal sialic acid of the oligosaccharide moiety by neuraminidase is a prerequisite for the extensive self–degradation of MT1–MMP. These data suggest that the terminal strongly negatively charged sialic acid is likely the most important functional component of the oligosaccharide cassette in MT1–MMP and that its presence, very likely, restricts the access of the catalytic domain to the hinge region and to the autolytic cleavage site. All together, our data are in agreement with and significantly extend the observations of many other laboratories and present a model where O–glycosylation regulates the autocatalysis of the enzyme and through this mechanism, the presentation and the functional activity of MT1–MMP in many cancer cell types.

Keywords: MT1–MMP, O–glycosylation, Self–proteolysis
Endogenous Upregulation of tPA Contributes To Histopathology After Cerebral Hypoxia/Ischemia In The Newborn Through LRP and ERK MAPK.

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Introduction: Perinatal cerebral hypoxia/ischemia has many causes, unclear pathophysiology, no specific treatment and poor outcome. Neonatal stroke has been estimated to occur in as many as 1 in 4000 births. In newborns with stroke, complications such as hypoxia/ischemia (H/I) are common. The only FDA approved treatment for stroke is tPA. Nonetheless, in addition to its salutary role in reperfusion, tPA contributes to excitotoxic neuronal cell death. Our previous research shows that uPA is upregulated after H/I and causes phosphorylation (activation) of the ERK isoform of mitogen activated protein kinase (MAPK) in a low-density lipoprotein (LRP) receptor dependent process. LRP mediates plasminogen activator (PA)-dependent vasoreactivity and the PA inhibitor-derived peptide EEIIMD blocks tPA and uPA vasodilation without altering catalytic activity or clearance. Here, we investigated the role of endogenous tPA, LRP, and ERK MAPK in histopathology after H/I.

Methods: Hypoxia (PaO2 of 35 mm Hg; 10 min) followed by global cerebral ischemia (elevated ICP; 20 min) was produced in newborn pigs. CSF tPA was quantified by ELISA. H&E staining was used to investigate histopathology and brain water content (BWC) was used as an index of edema 4h after H/I. Results: The concentration of tPA in the CSF increased from 41 ± 4 to 207 ± 15 ng/ml within 4h of H/I. Histopathology was graded blindly on H&E staining sections using a 3+ scale of severity. Neuronal appearance was normal in the hippocampal CA1 region in sham animals (--), many neurons with pyknosis were seen in the CA1 region after H/I (2–3+), but the appearance of the neurons in the CA1 region (-- ) remained normal in the injured animals that had received treatment with either the LRP antagonist RAP or the ERK MAPK antagonist U 0126 30 min prior to H/I (n=3). There were no histologic changes in the parietal cortex of sham animals (--), small hemorrhage with some edema (2+ to 3+) and high density of neurons with pyknosis following H/I (3+), but few neurons with pyknosis (+), and a modest number of neurons with pyknosis (2+), but no hemorrhage and edema (--) in the parietal lobe of animals that had been pretreated with RAP or U 0126 (n=3). BWC was elevated from 78.8 ± 0.8 % to 83.3 ± 0.2% after H/I and blunted (80.6 ± 0.3%) by EEIIMD (P<0.05, n=5). Intravenous administration of exogenous tPA (2 mg/Kg) prior to H/I increased BWC above that observed with H/I alone (85.6 ± 0.3%), which was blocked by EEIIMD (80.8 ± 0.3%) (P<0.05, n=5).

Conclusions: These data show that upregulation of tPA contributes to CNS histopathology after H/I through LRP and ERK MAPK and they identify a novel approach to ameliorate tPA-mediated injury.

Keywords: cerebral hypoxia/ischemia, mitogen activated protein kina, histopathology
Loss of p75 neurotrophin receptor exacerbates the effects of plasminogen deficiency

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Plasmin(ogen), through the degradation of fibrin matrices, plays a key role in regulating tissue fibrosis. Plasminogen deficiency (plg−/−) in vivo leads to liver degeneration, increased wasting and mortality due to excessive fibrin deposition (Bugge et al., 1996, Cell, 87:709–19). However, the cellular and molecular mechanisms that mediate the fibrin–induced pathology in the plg−/− mouse are not fully understood. Here we show that p75 neurotrophin receptor (p75NTR), a TNF receptor superfamily member upregulated in human fibrotic and cirrhotic liver exclusively by hepatic stellate cells (HSCs), regulates HSC differentiation and controls liver pathology and wasting in the plg−/− mouse. Mice deficient for both plasminogen and p75NTR (plg−/− p75NTR−/−) show an exacerbated disease state compared to the plasminogen knockout alone, displaying severe wasting and high mortality as early as eight weeks of age, as well as enhanced dystrophic liver calcifications and increased liver cell apoptosis. Moreover, genetic depletion of p75NTR leads to a decrease in hepatocyte growth factor (HGF) levels and inhibits hepatocyte proliferation in the plg−/− mouse. After excluding changes in fibrin deposition as the cause of the exacerbated liver disease in the double knockout mice, we examined the effects of p75NTR on HSC differentiation. After liver injury or disease HSCs differentiate into matrix-producing myofibroblasts that support hepatocyte proliferation during liver regeneration. HSCs isolated from p75NTR−/− mice fail to differentiate to myofibroblasts in vitro, as suggested by a dramatic reduction in both cell size and expression of alpha-smooth muscle actin, a stress fiber marker present only in myofibroblasts. Moreover, inhibition of p75NTR/Rho signaling in wild-type HSCs results in impaired HSC differentiation. In a co-culture system of HSCs with hepatocytes, p75NTR−/− HSCs do not support hepatocyte proliferation when compared to wild-type HSCs. Strikingly, the inability of the p75NTR−/− HSCs to support hepatocyte proliferation is rescued by addition of HGF. Overall, our results identify p75NTR/Rho signaling as a novel mechanism for the regulation of liver pathology in the plg−/− mouse by controlling HSC differentiation that supports regenerative processes in the diseased liver. Supported by NIH/NINDS R01 grant NS051470 to KA, and the UCSD Pharmacology NIH Training Grant ST32-GM07752 to MP.

Keywords: plasminogen, neurotrophin receptor, liver repair

ID: 64*
Fibrin/Mac-1 interactions induce microglia activation and regulate relapsing paralysis in central nervous system autoimmune disease

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In multiple sclerosis (MS), fibrin deposition temporally and spatially correlates with inflammatory demyelinating plaques. Although the presence of extravascular fibrin(ogen) at sites of inflammatory demyelination in MS has been documented by pathologists for decades, the molecular and cellular mechanisms of fibrin action in CNS pathogenesis have not been investigated. Here we show that fibrin signals through the Mac-1 (CD11b/CD18) integrin receptor to directly activate microglia and regulate the progression and severity of inflammatory demyelination. Fibrinogen directly activates microglia, the brain’s resident macrophages, resulting in dramatic cytoskeletal rearrangements and increased phagocytosis through a process involving the activation of Akt and Rho. Use of an antibody that inhibits the binding of fibrin to Mac-1 or the PI3K inhibitor, LY294002, blocks fibrinogen-induced microglia activation and phagocytosis. Pharmacologic depletion of fibrin using the snake venom ancrd in remitting-relapsing Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of MS, results in reduced activation of CD11b-positive microglia cells and effectively reverses relapsing paralysis. The importance of the Mac-1–fibrin(ogen) interaction to microglial activation and demyelination was further established through detailed studies of Fib gamma390–396A mice that express a form of fibrinogen that retains full clotting function but lacks the Mac-1 binding motif previously identified within the C-terminus of the fibrinogen gamma chain. The loss of the Mac-1 binding site within the fibrinogen gamma chain strongly attenuated microglial activation and diminished inflammatory demyelination in EAE. Our study provides a cellular and molecular definition of the role of fibrin in CNS pathogenesis and shows that fibrinogen-mediated microglia activation regulates the progression and severity of inflammatory demyelination. Targeting fibrin/Mac-1 interactions could represent a potential therapeutic strategy for MS with potential applications for other neuroinflammatory diseases associated with blood–brain barrier disruption and microglia activation. Supported by NIH/NINDS R01 Grant NS052189 and NMSS RG 3782–A–2 (KA), and NMSS Postdoctoral Fellowship FG 1582–A–1 (RA).

Keywords: Inflammation, Fibrinogen, Multiple Sclerosis
Fibrin deposition and axonal damage in spinal cord injury

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Regeneration failure of adult mammalian central nervous system (CNS) neurons is not due to their intrinsic inability to regenerate, but rather to the non-permissive CNS environment. Diseases of the nervous system associated with blood-brain barrier disruption, such as spinal cord injury (SCI) are associated with extensive leakage of blood components into the nervous tissue. However, the effects of the blood in the inhibition of CNS neuron regeneration have not been examined. We have previously shown that fibrin, derived from the blood protein fibrinogen, is an inhibitor of nerve regeneration in the peripheral nervous system (Akassoglou et al., 2002, Neuron 33: 861–875). Here we show the effects of fibrin in a model of CNS injury. First, we examined the temporal and spatial deposition of fibrin after SCI, in a dorsal column wire knife SCI rat model. Fibrin deposition rapidly increased in injured spinal cord parenchyma surrounding the lesion site and peaked at day 7. As expected, fibrin was undetectable in uninjured spinal cord. Moreover, fibrin deposition persisted even at 8 weeks, suggesting a decreased ability of the injured lesion site to clear fibrin. Double immunofluorescence confocal microscopy showed co-localisation of fibrin with IBA-1+ activated microglia/macrophages, while GFAP+ astrocytes were surrounding the fibrin deposits. Interestingly, fibrin deposition co-localized with the areas of SMI32+ degenerated axons, while GAP43+ regenerated axons were residing outside of the fibrin deposits. Taken together, our data identify that fibrin temporally and spatially correlates with axonal damage after spinal cord injury. Data on the molecular and cellular mechanisms for fibrin functions on neuronal cells during SCI will be presented.

Keywords: Fibrinogen, Neuron, Spinal cord injury
The tissue-type plasminogen activator/plasminogen system induces alterations in cortical neuron organization and survival

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Tissue–type plasminogen activator (t–PA) is abundantly expressed throughout the central nervous system where it exerts pleiotropic effects. In this study we show that the plasmin generated by t–PA on mouse cortical neurons, and the subsequently activated MMPs, degrade the extracellular matrix (ECM) and lead to loss of cell adhesion. Despite this detachment, the neurons remained associated forming aggregates interconnected by fasciculating fibers. Plasmin was indeed unable to dissociate these cell aggregates, in contrast to other adherent cell types that are detached and fully dissociated by plasmin. The formation of neuronal aggregates could not be prevented neither by mAbs that specifically block the cell adhesion molecules N–cadherin and L1 nor by the integrin antagonists RGDS and GRGDS peptides, suggesting that neuronal interconnections supporting aggregation, are not susceptible to plasmin or to inhibitors of these cell adhesion molecules. We explored the level of activation of different signaling kinases during this process. Plasmin–induced cell detachment and aggregation were concomitant with Src family kinases (SFK) activation. However, inhibition of SFK activation did not affect plasmin formation nor its consequences on neuron organization and viability. A plasmin(ogen) concentration– and time–dependent decrease in neurons viability was also observed as compared to control cells. In conclusion, we show that t–PA–mediated plasminogen activation and subsequent ECM degradation, induces alterations in cortical neurons organization and survival. Both reorganization and death of neurons induced by plasmin in vitro may be of biological relevance as t–PA is abundantly found in the CNS and in vivo studies have demonstrated a role of the plasminogen activation system in both neuronal plasticity and survival.

Keywords: cell detachment, neuronal aggregation, cell surface plasmin formation

* selected for oral presentation
† not represented at ISFP Congress
Neutralizing the neurotoxic effects of exogenous and endogenous tPA

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The clinical use of tissue-type plasminogen activator (tPA) in the treatment of stroke is profoundly constrained by serious side effects, including intracranial hemorrhage. We have previously reported that traumatic brain injury increases the level of tPA in the CNS and that tPA decreases cerebral vascular resistance in rats and piglets. We also reported that PAI–1 and a PAI–1 derived peptide hexapeptide (EEIIMD) corresponding to amino acids 350–355 of plasminogen activator inhibitor inhibit tPA–mediated signal transduction without compromising its catalytic activity or clearance. We now present data to show that the deleterious effects of tPA on cerebral edema and intracranial bleeding are separable from its fibrinolytic activity and can be neutralized. EEIIMD decreased tPA–induced increase in infarct size in rats by more than 65\% (\textit{n}=12, \textit{P}<0.01) and the prevalence of intracranial bleeding by >90\% (\textit{P}=0.0002) in a mechanical model of stroke. EEIIMD also reduced infarct size induced by cerebrovascular emboli more than 3–fold (\textit{n}=6, \textit{P} <0.01). EEIMD also neutralized the deleterious effect of tPA after head trauma in pigs by reducing brain water content, brain edema and neuronal loss in the parietal cortex and hippocampus (\textit{n}=6, \textit{P}<0.05, each). These studies indicate a means to reduce the neurotoxic effects of tPA without compromising its fibrinolytic activity through the use of selective antagonists and novel tPA formulations.

\textbf{Keywords:} tissue–type plasminogen, Neurotoxic effects, Brain injury
Tissue-Type Plasminogen Activator-Plasmin Cascade in Experience-Dependent Plasticity of Visual Cortex

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In the mammalian central nervous system, the tissue-type plasminogen activator (tPA) is thought to participate in a variety of postnatal developmental events and neuronal plasticity, such as cell migration, experience-dependent circuit refinement and learning. Here, we report specific role of tPA concerning morphological rearrangement in mouse visual cortex during a so-called critical period early in life. The shift of ocular dominance (OD) distribution following sensory deprivation is one of the most well-characterized models for experience-dependent plasticity accompanied by morphological rearrangement. We are able to quantify the functional plasticity using extracellular unit recording in vivo. It is, however, largely unknown how this rapid physiological plasticity is coupled to morphological refinements (Antonini et al., 1999). Extracellular proteolysis is one mechanism that can remodel neuronal circuits by degradation of extracellular matrix components. We, therefore, hypothesized that the tPA protease system acts to degrade cell surface receptors, cell adhesion molecules or extracellular matrix proteins, thus permitting morphological changes and synaptic remodeling dependent on visual experience. Indeed, physiological plasticity is reversibly impaired in tPA knockout mice (tPA KO) throughout life (Mataga et al., 2002). To explore direct effects of tPA upon neurite structure in the visual cortex, we labeled cells by randomly delivering lipophilic dye-coated particles (DiI) into fixed slices of tPA KO and wild-type (WT) mice. The number of protrusions, including spines and filopodia was counted along the apical dendrite of typical excitatory neurons in the binocular zone of visual cortex. In both genotypes, protrusions on the apical dendrite of layer 2/3 pyramidal cells increased steadily in number with postnatal age with no simple relation to the critical period profile. However, brief MD for 4 days led to pruning of spines on apical dendrites only in the binocular zone of WT mice during the critical period. Proteolytic activity by tPA conversely declined with age in visual cortex, and increased transiently upon MD again only in young WT mice. Importantly, spine loss was impaired in tPA KO mice but partially restored starting from dendritic segments nearest the cell body by exogenous tPA injections, similar to previous findings for physiological changes. Deletion of the tPA substrate plasminogen mimicked the impaired physiological plasticity observed in tPA KO mice after brief MD. The tPA–plasmin axis may, thus, mediate rapid structural rearrangement underlying experience-dependent plasticity.

Keywords: ocular dominance, critical period, structural rearrangement
Regulation of tPA and PAI-1 gene expression in human astrocytes

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Background We have reported that the t-PA -7,351C>T enhancer polymorphism is functional at the level of transcription. In vitro studies on endothelial cells also showed that this variant influences expression of the endogenous tPA gene in response to a wide spectrum of stimuli, including retinoic acid (RA) and PKC activators. We have also shown that the mutant “low expression” allele is associated with reduced vascular tPA release in vivo and, in line with the thromboprotective role of tPA, an increased risk of myocardial infarction was observed in carriers of this allele. In contrast, in a large study on ischemic stroke we did not detect any association for the tPA polymorphism alone, whereas the tPA -7,351CC/PAI-1 4G4G genotype combination (i.e. high tPA and PAI-1 expression) was protective. A potential explanation for this discrepancy may be that t-PA makes neurons more vulnerable to excitotoxic damage and that this deleterious effect of tPA can be neutralized by PAI-1, synthesized by astrocytes. The aim of the present work was therefore to characterize tPA and PAI-1 gene expression in astrocytes.

Methods Native human astrocytes were stimulated by various agonists including retinoic acid (RA), PKC activators (PMA, histamine), PKA activator (forskolin), growth factors (VEGF, BDNF, TGF-alfa, TGF-beta) and cytokines (IL-1beta, IL-6, TNF-alfa). Stimulations were performed for 3, 6 and 20 hours (n=6 in each group). tPA and PAI-1 mRNA expression and protein secretion was determined by RT-PCR and ELISA, respectively.

Results Treatment with RA or PMA both induced a 3-fold up-regulation of tPA mRNA expression and protein secretion after 20 h stimulation compared to DMSO control treated cells. When combined, a 9-fold induction was observed. A similar but weaker response was seen following treatment with histamine and none of the stimulations markedly affected the expression of PAI-1. Forskolin induced a strong down-regulation of both proteins that became more pronounced with time, in particular for PAI-1. Following stimulation with growth factors and cytokines, a general weak up-regulation of tPA and PAI-1 expression was observed at 3 and 6 h followed by a slight down-regulation at 20 h. When examining the molecular ratio of tPA and PAI-1 in the cell culture media following the various treatments, there was a shift toward a higher ratio after combined treatment with RA and PMA, while a shift towards a reduced tPA/PAI-1 ratio was observed in response to TGF-alfa, TGF-beta and IL-1beta.

Conclusion The results show for the first time that in astrocytes, like in endothelial cells, both RA and PKC activation induce a strong up-regulation of tPA and that the response to these two stimuli is synergistic. PAI-1 expression is unaffected. In contrast, PKA activation reduces both tPA and PAI-1 expression. Growth factors and cytokines induce a time-dependent response pattern of both proteins with a slight up-regulation at early time-points followed by a down-regulation. We will now proceed by investigating whether these responses are genotype-dependent.

Keywords: Gene expression, tPA, PAI-1
Tissue-type plasminogen activator can promote NMDA-induced neuronal stimulation via LDL receptor and plasmin-dependent mechanisms.

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Glutamate is the main excitatory neurotransmitter of the mammalian brain. The efficacy of glutamatergic neurotransmission is not fixed, but can vary in a stimulus-dependent manner – a neural phenomenon known as synaptic plasticity that underlies learning and memory formation. Current evidence has identified t-PA as a modulator of synaptic plasticity. The glutamate-binding NMDA receptor (NMDAR) plays a central role in synaptic plasticity. Several recent reports have highlighted the NMDAR as the cell-surface receptor through which t-PA can exert its neuromodulatory effects. The mechanism by which t-PA influences the NMDAR, however, has been the subject of much controversy. To date, three models explaining the ability of t-PA to alter NMDAR-mediated processes have been put forward: (1) direct cleavage of the NR1 subunit of the NMDAR; (2) a non–proteolytic interaction with the NR2B subunit of the NMDAR; and (3) a signaling cross-talk mechanism between the NMDAR and a Low-Density Lipoprotein Receptor (LDLR). The ability of t-PA to modulate NMDAR function is exemplified by its ability to potentiate NMDA-induced calcium flux in primary neuronal cultures. Here we show that this action of t-PA requires proteolysis as inactive t-PA fails to potentiate NMDA-induced calcium flux. Although the NR1 subunit has been suggested to be the operative substrate for t-PA, our western blot analyses provide no evidence for t-PA–mediated NR1 subunit cleavage. This was assessed by adding t-PA to either intact neuronal cultures or to brain extracts and used both N- and C-terminal anti-NR1 antibodies. We also exclude plasminogen as being the operative substrate behind the t-PA–mediated potentiation. Rather, our findings suggest that the enhancement of NMDAR function by t-PA is mediated by a LDLR, since the potentiating effect is abolished by the LDLR pan–ligand blocking agent, RAP (receptor–associated protein). That t-PA requires proteolytic activity and an LDLR suggests a functional relationship between t-PA, the NMDAR, a LDLR and an unknown substrate. This finding is consistent with earlier reports that a LDLR mediates the facilitation of NMDAR–dependent synaptic plasticity by t-PA. Although plasmin plays no role in the potentiating effect of t-PA on NMDA–induced calcium flux, high plasmin concentrations (>125nM) were found to trigger a pronounced and reversible rise in neuronal intracellular calcium concentrations. Furthermore, the NMDAR antagonist, MK801, abolished plasmin–induced calcium flux, indicating that this effect is NMDAR–dependent. Moreover, this effect of plasmin was correlated with discrete cleavage of the NR1 subunit. Hence, plasmin and t-PA have distinct functional and biochemical effects on the NMDAR. On the basis of this data, we propose that t-PA influences the NMDAR via two mechanisms. The first involves the plasmin–independent, but LDLR–dependent, potentiation of NMDA–induced calcium flux. The second mechanism is plasmin–dependent, whereby t-PA–mediated plasmin generation triggers NMDAR activation. To confirm these conclusions we are presently determining whether t-PA and plasmin can independently alter the electrophysiology of the NMDAR.

Keywords: t-PA, neuron, NMDA

* selected for oral presentation  † not represented at ISFP Congress
Unconventional translation initiation of trypsinogen 4 at a CUG codon with an N-terminal leucine: a possible means to regulate gene expression

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Chromosomal rearrangements led to the formation of a primate specific gene PRSS3 in chromosome 9. It encodes, due to alternative splicing, both mesotrypsinogen and trypsinogen 4. While mesotrypsinogen has been known as a pancreatic protease, the chemical nature and biological function of trypsinogen 4 has not been explored yet. According to the prediction isoform A and B of trypsinogen 4, dependent on the use of either an AUG or a CUG translation initiation site, would contain a 72 or a 28 amino acid leader peptide, respectively. For the first time, our present studies provide evidence for the N-terminal amino acid sequence of trypsinogen 4 and the possible mechanism of the expression of this protein in human brain and transiently transfected cells. We raised monoclonal antibodies against a 28-amino acid synthetic peptide representing the leader sequence of isoform B and against recombinant trypsin 4. By using these antibodies we isolated and chemically identified trypsinogen 4 from the extracts of both post mortem human brain and transiently transfected HeLa cells. Our results show that Isoform B with a leucine N-terminus is the predominant (if not exclusive) form of the enzyme in post mortem human brain and that the same isoform is also expressed in transiently transfected cells. Based on our studies on the expression of a series of trypsinogen 4 constructs in two different cell lines we propose that unconventional translation initiation at a CUG with a leucine N-terminus may serve as a means to regulate protein expression.

Keywords: human brain, translation, gene expression
Recurrent stroke due to intra-luminal carotid thrombus in a patient with active multiple myeloma

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Intra-luminal carotid thrombus is rare in the absence of significant stenosis. The evidence base to help guide acute management of such patients is sparse due to the rarity of this condition. A 39-year-old woman presented with a dominant hemisphere stroke. Carotid duplex, 24-hour cardiac monitor and transesophageal echocardiography did not reveal a mechanism, but active multiple myeloma was found. She underwent chemotherapy for myeloma, and aspirin and a statin were commenced for secondary stroke prevention. She represented five years later with recurrent stroke in the same vascular territory, raising the possibility of a fixed arterial lesion. The myeloma had initially responded to treatment but she had recently been diagnosed with new bony lesions. Dynamic neck MRA demonstrated a large intra-luminal thrombus associated with only minor luminal irregularity within the left internal carotid artery (ICA) just beyond the bifurcation. She was considered to be at high risk for recurrent artery-artery embolism. Angiography confirmed the presence of an intra-luminal filling defect. An attempt was made to capture and remove the intra-luminal thrombus. This was unsuccessful, thus a stent was deployed from the common carotid artery into the ICA, flattening the thrombus against the vessel wall. This achieved a good radiological result with no residual stenosis or filling defect. She improved clinically to her baseline level and was discharged on combination antiplatelet therapy and a statin. Intra-luminal ICA thrombus is rare and usually associated with severe atheromatous disease. Reports of spontaneous carotid thrombus suggest that affected patients are younger and that some have an underlying hematological disorder. While our patient did not have significant carotid stenosis, there was some minor luminal irregularity in the left ICA. This may reflect mild atheromatous disease from smoking. Given that both infarcts were in the same vascular territory and that there was a five-year interval between events, we postulate that the underlying luminal irregularity was sufficient to elicit superimposed thrombus formation only while her myeloma was active, while she had a concomitant prothrombotic state. There is little evidence on which to base acute management. Urgent surgery is associated with significant risk of perioperative stroke. The glycoprotein IIb/IIa receptor antagonist Tirofiban has been used to dissolve intra-luminal thrombus, however carries significant risk of hemorrhage particularly in the setting of carotid stenting. There are several endovascular devices available for thrombectomy, but one study found that several were associated with distal embolisation. While the attempt at endovascular thrombectomy in our patient was unsuccessful, using the stent as a mechanical means of flattening the thrombus against the vessel wall gave a good angiographic result and importantly was not associated with any neurological complications. An endovascular device has recently been approved by the FDA for foreign body extraction and intracranial thrombectomy in the setting of acute cerebral ischemia. This type of device may offer a new method of managing such patients, but until there is more experience with new endovascular embolectomy devices, angioplasty and stenting of intra-luminal carotid thrombus may offer a safe alternative.

Keywords: multiple myeloma, stroke, stent
p75 Neurotrophin Receptor Regulates Fibrinolysis via a cAMP/PKA Pathway

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Clearance of fibrin through proteolytic degradation is a critical step of matrix remodeling that contributes to tissue repair in a variety of pathological conditions, such as stroke, atherosclerosis and pulmonary disease. However, the molecular mechanisms that regulate fibrin deposition are not known. Here, using both in vivo and in vitro systems, we report that a TNF receptor superfamily member upregulated after tissue injury, the p75 neurotrophin receptor (p75NTR), blocks fibrinolysis by downregulating the serine protease, tissue Plasminogen Activator (tPA), and upregulating Plasminogen Activator Inhibitor–1 (PAI–1). We have demonstrated that elevation of cAMP in p75NTR–expressing cells is able to restore fibrinolysis. These results implicate the cAMP pathway as a potential link between p75NTR and the regulation of plasminogen activation. Moreover, mice deficient for p75NTR show a dramatic increase of proteolytic activity and a reduction in fibrin deposition in both sciatic nerve after injury and in lung tissues after lipopolysaccharide (LPS)–induced fibrosis. Our results therefore demonstrate a novel pathogenic mechanism by which p75NTR perpetuates scar formation after injury both within and outside of the nervous system, resulting in an environment hostile for regeneration and repair.

Keywords: tissue plasminogen activator, PAI–1, fibrinolysis
Plasminogen Activator is Necessary for Spinal Cord Synaptic Plasticity

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In addition to its role in the vascular system, plasminogen activator (PA) is involved in neural development, excitotoxic cell death, and has been implicated in aspects of cerebral synaptic remodeling associated with cerebellar motor learning, visual cortex ocular dominance columns, and hippocampal & cortico–striatal LTP & LTD. We have explored the possibility that PA may also play a role in synaptic plasticity in the spinal cord. The crossed phrenic phenomenon (CPP) describes respiratory functional plasticity that arises following spinal cord injury; whereby, phrenic motoneuron drive to the diaphragm is restored following activation of “functionally ineffective” medullary respiratory neuron synapses on phrenic motoneurons (PMN). Synaptic remodeling is thought to occur during the characteristic delay period following spinal cord injury before the CPP becomes functional. The mechanisms underlying this synaptic plasticity are not well-defined. Our ultimate aim is to understand the underlying molecular mechanisms of this functional recovery using a mouse model amenable to a molecular genetic approach. Using electromyographic (EMG) recordings from the diaphragm, we examined the inter-operative delay time between spinal cord hemisection and contralateral phrenicotomY required for diaphragm response, as compared to animal death from asphyxia at zero time. A critical 1–2 hr window is required for this synaptic plasticity. In situ hybridization shows that uPA and tPA mRNAs are rapidly induced in C4–5 ventral spinal cord neurons in the ipsilateral phrenic nucleus compared to the contralateral PMN and sham controls, with elevated PA protein at 1 hr post-hemisection. The specific and concomitant induction of PA suggests a role in CPP spinal cord plasticity. This is confirmed by the failure of most uPA knockout and plasminogen knockout mice to acquire the CPP by 6 hr post-hemisection, a time when all tPA knockout and wildtype mice show a CPP, as well as a very strong synaptic response seen in >95% of wildtype mice. Thus, suggesting future potential therapeutic uses for PA in spinal cord injury.

Keywords: Spinal cord injury, plasminogen activator, synaptic plasticity
Development and Characterization of a Murine Model of Thrombotic Stroke

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The current generation of thrombolytic agents such as tissue-type plasminogen activator (tPA) only benefit a limited number of patients with ischemic stroke, and the development of new therapies for the treatment of stroke depends upon understanding the unique characteristics of hemostasis within the brain. The limited benefit of tPA seems to be due in part to the unique activities that tPA has in the brain beyond its well established role as a fibrinolytic enzyme. These recently described effects of tPA present unique challenges for the use of thrombolytic therapy in ischemic stroke, and suggest that ideal treatments should simultaneously promote the reestablishment of vascular patency, inhibit the development of cerebral edema, and provide direct neuroprotection. Conventional stroke models, such as the suture model or the ligation / cauterization model, are not suitable for these types of studies, and in order to identify new treatments for stroke that combine thrombolytic and neuroprotective agents it is necessary to develop a stroke model that permits thrombolytic treatment. To achieve this goal we have developed a novel murine model of thrombotic stroke that permits combined treatment with thrombolytics as well as with neuroprotective agents. The model is based on photothrombosis with the photoactivatable dye Rose Bengal. Strokes are induced specifically in the middle cerebral artery (MCA) by the local activation of intravenous Rose Bengal with a cold laser. This results in free radical formation which damages the endothelium locally, leading to platelet aggregation, activation of the coagulation cascade, and occlusion. Laser Doppler analysis of tissue perfusion in an area of the cerebral cortex 1.5 mm dorsal median from the bifurcation of MCA, monitored continuously for two hours after the initiation of photothrombosis demonstrates that the overall tissue perfusion drops over the first forty minutes after photoactivation resulting in complete occlusion with an average time to occlusion of 54 ± 19 minutes. The strokes induced by the photothrombosis were analyzed after 6 and 24 hours using MRI, and at 24 hours by TTC staining and histology. These data indicate that the strokes are largely cortical, with a typical looking ischemic border (penumbra). Analysis of infarct volumes demonstrates that the model is highly reproducible, with infarct size increasing approximately 43 % from 6 to 24 hours, going from an average volume of 37 ± 5 microL at 6 hours to an average volume of 53 ± 1 microL by 24 hours. The final infarct volumes obtained by MRI at 24 hours were also in good agreement with infarct volumes calculated from TTC analysis of the same mice immediately after the 24 hour MRI analysis, which gave an average infarct volume of 51 ± 4 microL. We have also examined whether these photochemical induced thrombi can be treated with thrombolytic therapy in vivo, and these data indicate that after stable thrombus formation tPA infusion can induce significant clot lysis and restore cerebral blood flow. Together, these data indicate that this photothrombotic model should permit effective analysis of combined thrombolytic and neuroprotective therapies in vivo.

Keywords: Stroke, thrombolytic, neuroprotection
MODULATION OF NEUROTRANSMITTER RELEASE FROM CATECHOLAMINERGIC CELLS BY THE BINDING OF PLASMINOGEN TO CELL SURFACE ACTIN FORMS

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Recent reports have documented a novel role for the plasminogen activation system in the regulation of neurotransmitter release. Prohormones, secreted from cells within the sympathoadrenal system, are processed by plasmin to bioactive peptides that feed back to inhibit secretagogue-stimulated release. Catecholaminergic cells of the sympathoadrenal system are prototypic prohormone secreting cells. Processing of prohormones by plasmin is enhanced in the presence of catecholaminergic cells and the enhancement requires binding of plasmin(ogen) to cellular receptors. Consequently, modulation of the local cellular fibrinolytic system of catecholaminergic cells causes substantial changes in catecholamine release. However, mechanisms for enhancing prohormone processing and cell-surface molecules mediating the enhancement on catecholaminergic cells have not been investigated. Here we show that plasminogen activation was enhanced on catecholaminergic PC12 cells and bovine adrenal chromaffin cells 6.5-fold and 10-fold, respectively. Carboxypeptidase B treatment decreased cell-dependent plasminogen activation by >90%, suggesting that the binding of plasminogen to proteins exposing carboxyl-terminal lysines on the cell surface is necessary for promotion of plasminogen activation. We identified catecholaminergic plasminogen receptors required for enhancing plasminogen activation, using a strategy that combined targeted specific proteolysis using carboxypeptidase B with a proteomics approach employing 2-dimensional gel electrophoresis, radioligand blotting and tandem mass spectrometry. Two major plasminogen binding proteins that exposed carboxyl-terminal lysines on the cell surface were identified as beta/gamma-actin. An anti-actin monoclonal antibody inhibited cell-dependent plasminogen activation by 31.4 ± 0.3% (P<0.001) and concomitantly enhanced nicotine-dependent catecholamine release by 40.1 ± 0.4%, (P<0.001). Our results suggest that cell surface–expressed forms of actin bind plasminogen, thereby promoting plasminogen activation and increased prohormone processing leading to production of inhibitory bioactive peptides and consequent inhibition of neurotransmitter release.

Keywords: plasminogen receptor, catecholamines, chromaffin cells
Role of the Plasminogen Activation System in Hippocampal Neuritogenesis

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A major role for the plasminogen activation system in neuronal function has been established in the literature. Here we report that the plasminogen activation system regulates neuritogenesis of hippocampal neurons. When 0.5 mM plasminogen was added to primary cultures of murine hippocampal neurons, the number of neurite positive cells after 24 hr of culture increased from 35.37 ± 2.2% to 61.09 ± 3.8% (p<0.05) compared to untreated neurons. After 72 hr of culture, the average neurite length increased from 68.34 ± 0.6 mm to 110.3 ± 1.4 mm (p<0.001) by plasminogen treatment. Similar results were obtained with embryonic rat hippocampal neurons. Furthermore, differential immunostaining of rat hippocampal neurons indicated that the average axon length increased from 51.8± 6.24 mm to 106.6 ± 6.4 mm (p<0.01) and the average dendrite length was increased from 42.33 ± 4.5 mm to 87.7 ± 5.6 mm (p<0.01). Conversely, the growth cone areas of the hippocampal neurons were markedly decreased following plasminogen treatment, consistent with the idea that larger growth cones are often stationary and contribute less to neurite outgrowth. The stimulation of neuritogenesis by plasminogen was abolished in the presence of aprotinin, suggesting that the activation of plasminogen by endogenous plasminogen activators was required. Therefore, we examined neurite outgrowth in t-PA −/− mice. Neurite length and the number of neurite positive cells were not statistically different when comparing t-PA −/− vs wild type hippocampal neurons. However, application of amiloride, an inhibitor of u-PA, decreased neurite outgrowth from wild type hippocampal neurons [69.8 ± 2.5 mm vs 43.28 ± 2.5 mm (p<0.05) for untreated vs amiloride-treated, respectively]. Interestingly, the inhibitory effect of amiloride on the length of t-PA −/− neurons (64.33 ± 3.6 mm vs 33.67 ± 1.4 mm for untreated t-PA −/− cells vs amiloride-treated t-PA −/− cells, respectively) was significantly greater (p<0.005) than the effect on wild type neurons. There was a statistically significant difference between amiloride treated wild type (43.28 ± 2.5 mm) and amiloride treated t-PA −/− neurons (33.67 ± 1.4 mm (p<0.05). These results suggest that both neuronal u-PA and neuronal t-PA participate in hippocampal neurite outgrowth. In addition, a direct interaction of plasminogen with the hippocampal neurons appeared to be required because e-aminocaproic acid (EACA) abolished the enhancement of neurite outgrowth by mouse hippocampal neurons following the addition of plasminogen [50.72 ± 3.8 mm vs 139.4 ± 6.3 mm (p<0.005) for EACA + plasminogen vs plasminogen alone, respectively]. EACA also decreased neurite positive cells in untreated hippocampal neurons [86.66 ± 5.9% vs 68.75 ± 4.5% (p<0.05) for untreated vs EACA treated, respectively], suggesting that endogenous neuronal plasminogen is required for optimal neuritogenesis. Accordingly, we found that neurite outgrowth was significantly decreased in plasminogen deficient hippocampal neurons, compared to wild type controls [43.38 ± 1.1 mm vs 62.18 ± 2.25 mm (p<0.02) for plasminogen deficient vs wild type hippocampal neurons, respectively]. These observations suggest that endogenous plasminogen is required for optimal hippocampal neuritogenesis. Furthermore, active plasmin is required. Finally, sufficient plasminogen activation can be accomplished by either endogenous t-PA or endogenous u-PA.

Keywords: plasminogen activation system, neuritogenesis, hippocampal neuron
New Co-factors

**ID:** 133*

**Beyond proteomics: Amyloid-like structures, independent of amino acid sequences, as activators of proteolysis.**

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Recently, it has been discovered that the finger domains of tissue-type plasminogen activator (tPA) and Factor XII strongly bind to amyloid-like structures of multiple beta-sheets of proteins: cross-beta structures. This binding occurs in cross-beta structures of several proteins indicating a common feature, independent of amino-acid sequences, visualised by X-ray, and underlying the mechanism. These bindings induce activation of the tPA-plasminogen and the factor XII-route. It has so far been demonstrated that cross-beta structures can be induced in proteins by several mechanisms creating the triggers for tPA and factor XII activation. These include: binding of proteins to negatively charged surfaces, fibrin formation, oxidation and glycation of proteins, amyloid formation and in vitro stresses of purification. For factor XII activation it could be demonstrated that only in the presence of surface-binding protein, factor XII becomes activated. It was verified that neutralisation of the cross-beta structure in these bound proteins inhibit the tPA and factor XII activation. It changes the context of physiological and pathophysiological relevance of the proteolysis processes associated with tPA and factor XII. Not only chemically modified proteins, but also merely conformationally changed proteins are involved. The consequences of this change in paradigm will be listed and discussed. It implies mechanisms beyond proteomics, involving amyloid-like structures. For physiology and pathophysiology it calls for re-evaluation of the role of tPA and factor XII in diseases involving misfolded proteins such as Alzheimer’s disease, amyloid deposition, diabetes and atherosclerosis.

**Keywords:** amyloid, tissue type plasminogen activa, Factor XII
Non-fibrinolytic Proteases

**ID:** 24

**LRP and avb3 integrin mediate tPA-activation of smooth muscle cells.**

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Tissue-type plasminogen activator (tPA) regulates vascular contractility through the low-density lipoprotein–related receptor (LRP) and this effect is inhibited by plasminogen activator inhibitor–1. We now report that tPA–mediated vasocontraction also requires the integrin avb3. tPA–induced contraction of rat aortic rings is inhibited by the RGD peptide and by monoclonal anti–avb3 antibody. tPA induces binding of LRP to avb3 in vascular smooth muscle cells. The three proteins are internalized within 10 minutes causing the cells to become refractory to re-addition of tPA. LRP and avb3 return to the cell surface by 90 minutes restoring cell responsiveness to tPA. PAI–1 and the PAI–1–derived hexapeptide EEIIMD abolish the vasoconstrictive activity of tPA and inhibit the tPA–mediated interaction between LRP and avb3. PAI–1, RGD and antibodies to both LRP and avb3 inhibit the effect of tPA on Ca++ mobilization in SMC. These data indicate that tPA–mediated vasoconstriction involves the coordinated interaction of LRP with avb3. Understanding the mechanism underlying these interactions and the nature of the signals transduced may provide new tools to regulate vascular tone and other consequences of tPA–mediated signaling.

**Keywords:** tPA, LRP, Integrins
Purification and characterization of ancistron–Bu, a novel fibrinogen-clotting serine protease from Agkistrodon blomhoffii ussuriensis venom

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Snake venoms, particularly those belonging to the Crotalidae and Viperidae families, are known to contain proteases which act upon the different stages of blood coagulation. A number of these enzymes are termed thrombin–like enzymes, according to their ability to operate just like the most familiar of the many actions of thrombin, the capacity to interact directly with fibrinogen molecules, causing them to polymerize into fibrin. The fibrin monomers, produced by thrombin–like enzymes, are non–cross–linked, because many of these enzymes do not activate plasma factor XIII. Consequently, such abnormal fibrin are easily dispersible and more susceptible to plasmin proteolysis than thrombin–induced fibrin. Therefore, after intravenous administration thrombin–like enzymes can cause a rapid defibrinogenation. A novel fibrinogen–clotting enzyme (named ancistron–Bu) has been homogeneity purified from the Agkistrodon blomhoffii ussuriensis venom. This purification process consist of a two-step chromatographic separation on Blue Sepharose FF followed by a Sephadex G 25 gel filtration. Ancistron–Bu, obtained by this way, proved as homogeneous and show molecular weight approximately 13,5 and 27 kDa in reducing and non–reducing conditions of sodium dodecyl sulphate polyacrylamide gel electrophoresis respectively. The protein purity was about 99%. Its yield was 8,4% of total protein and purity increasing was 20,5 fold. This enzyme showed specific fibrinogen–clotting activity equivalent to 46,4 international thrombin units/mg. Unlike alpha–thrombin, ancistron–Bu split off fibrinopeptide A without releasing fibrinopeptide B from fibrinogen. The optimal pH range for the clotting activity of ancistron–Bu was 7,4–8,0. The hydrolytic activity of ancistron–Bu is strongly inhibited by diisopropyl fluorophosphate (it is obvious that this enzyme is serine protease), but heparin–antithrombin–III complex has a small effect on ancistron–Bu catalytic properties. This enzyme did not activate factor XIII and had not fibrinolytic and caseinolytic activities. Thrombin–specific substrate (D–Phe–Pip–Arg–p–nitroanilide) and protein C substrate (pyroGlu–Pro–Arg–p–nitroanilide) were most susceptible to hydrolysis by ancistron–Bu. In that time this enzyme did not showed any detectable amidolytic activity on the tested chromogenic substrates for plasmin and plasma factor Xa. Ancistron–Bu did not induce aggregation of washed normal platelets by itself, but caused plateletes aggregation in the presence of exogenous fibrinogen in a manner entirely different from that of thrombin. Ancistron time (like reptilase time) is a simple alternative to the thrombin time for rapid fibrinogen assay in samples containing heparin and is particularly useful in the assay of antithrombin–III where plasma can be prepared free of fibrinogen. The presence of fibrin degradation products, hypofibrinogenaemia and defects in fibrin polymerisation will prolong the ancistron time. Ancistron–Bu can also be used in preparing desAA–fibrinogen, the former for use as a fibrin stimulant in the functional assay of tissue plasminogen activator.

Keywords: Snake venom, Thrombin–like enzymes, Serine protease
Inhibition of an Aspartyl Protease: Kinetic and NMR Studies on Pepsin

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Aspartyl proteases play an essential role in the development of many diseases such as HIV and Alzheimer’s disease. Therefore the design of new and improved inhibitors is important in the treatment of such diseases. Pepsin is an aspartyl protease which has been widely studied and it is commercially available at large quantities. It is therefore ideal for the testing of new aspartyl protease inhibitors. Glyoxal inhibitors have been shown to effectively inhibit serine and thiol proteases. The interaction of glyoxal inhibitors with aspartyl proteases has not been studied by NMR. In this study we intend to determine whether glyoxal inhibitors are potent inhibitors of the aspartyl proteases. By using NMR to study the interaction of the proteinase with the glyoxal inhibitor the structure of the bound inhibitor can be determined. Initial kinetic studies were successful in determining the potency of a range of glyoxal inhibitors with Pepsin. The inhibitors tested were ZPheGlx, ZAlaPheGlx and ZAlaAlaPheGlx. To measure inhibition by the glyoxal inhibitors Pepsin was assayed using the substrates Z-His-Phe(NO2)-Phe-OMet and Phe-Ala-Ala-Phe(NO2)-Phe-Val-Leu-OM4P. These substrates were used to determine the Ki values of the glyoxal inhibitors. 13C enriched glyoxal inhibitors have been synthesized. A detailed NMR study of the interaction of the enriched glyoxal inhibitors with Pepsin has been undertaken. This study has allowed us to determine how the enriched glyoxal inhibitors are stabilised by Pepsin. We have also examined the effect of pH on the enriched enzyme-inhibitor species.
Application of In Vivo Labeling of Cysteine Cathepsin Protease activities:
Identification of Drug Inhibition Profiles in Mouse Tissues

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Cysteine cathepsins such as cathepsins B, K, L and S are potential pharmacological targets and anti-targets for various indications. Whole cell enzyme occupancy assays have been used to demonstrate the presence of catalytically active cathepsins in various cell lines and to determine inhibitor potencies against these cathepsin activities in their native lysosomal or endosomal environments. These assays employ a radiolabeled irreversible inhibitor (125I–BIL–DMK) which competes with the reversible test compound for the active site of the target enzyme. We have shown that basic cathepsin K inhibitors are more potent against both target and anti-target cathepsins in cell–based enzyme occupancy assays, than against each purified enzyme. This results from the ability of basic, lipophilic molecules to accumulate in acidic compartments, a phenomenon known as lysosomotropism. In contrast, non-basic cathepsin K inhibitors do not exhibit lysosomotropic properties. Here, we describe the development of an in vivo enzyme occupancy assay to determine whether the inhibition profile of basic and non-basic cathepsin inhibitors in vivo is the same as that observed in cell–based assays. This new approach allows the identification of active cathepsins in various mouse tissues, as well as the in vivo profile of inhibitors. Following p.o. dosing of the test compound, mice were dosed i.v. with the cell permeable probe 125I–BIL–DMK and tissues harvested 1 hour later. Fresh tissue lysates were fractionated by SDS–PAGE, dried, and visualized by autoradiography. Labeling of cathepsin B, L and S was confirmed by 2D gel analysis in liver, spleen, lung and kidney. By 1D-analysis, we observed that basic cathepsin K inhibitors gave increased competition of probe labeling of cathepsin B, L and S compared to non–basic inhibitors which is in accord with the lysosomotropism effects observed in whole cells. A non–basic selective cathepsin K inhibitor competed very weakly against cathepsin B, L and S in various mice tissues, whereas a non–basic cathepsin S inhibitor was able to block cathepsin S selectively in a dose dependent manner. This work demonstrates that selective, non–basic cathepsin K inhibitors can be differentiated from basic and/or non–selective inhibitors in terms of off–target activity in vivo.

Keywords: cathepsins, labeling, inhibitors
The interaction of active-site blocked thrombin and bivalirudin

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Introduction: Bivalirudin is a direct thrombin inhibitor which binds to the active-site and exosite-I of thrombin. The aim of this study was to characterize bivalirudin binding to active-site blocked thrombin (THi).

Methods: After IRB approval and written consents, blood was drawn from 6 healthy volunteers into 3.2 % citrate tubes. For thrombin generation (TG) experiments, platelet poor plasma (PPP) was obtained by centrifugation (2500 x g for 20 min at 10 °C). The effect of THi (0–250 ug/ml, Haematologic Technologies, VT) on TG in the presence or absence of bivalirudin (20 ug/ml, Medicines Company, MA) was assessed using Thrombinoscope™ (Synapse BV, Maastricht, the Netherlands), which measures the onset amount of thrombin based on the change in fluorescence signal produced by cleavage of substrate (Z-GGR-AMC HCl, Bachem, CA). The relipidated PPP reagent containing tissue factor (5 pM, Biodis, France) was used as an activator and the progress of reaction was monitored in 96 well plate. Similarly, the effect of THi (0–250 ug/ml) on clot formation was evaluated by thrombelastography (TEG®, Haemoscope, IL) using tissue factor activation (5 pM) in whole blood samples with or without bivalirudin.

Results: THi per se did not affect TG (no effect on lag time or thrombin peak), but bivalirudin–induced prolongation of lag time was shortened dose dependently (8.6 min with THi 250 ug/ml vs. 74.6 min with bivalirudin only). On TEG®, THi caused progressive delay of clot formation in a dose dependent manner (lag time from 7.4 +/- 2.6 with no THi to 23.0 +/- 0.3 min with THi 250 ug/ml). In the presence of bivalirudin (20 ug/ml), clot formation was faster with THi at 10 ug/ml (15.6 +/- 4.0 vs. 19.7 +/- 2.7 min without THi). Clot formation was still inhibited at higher concentrations of THi.

Conclusion: THi restores thrombin generation by binding to bivalirudin, but THi itself occupies thrombin–binding sites of platelets and fibrinogen, and therefore clotting is not recovered. Based on the requirement for high concentrations of THi for recovering thrombin generation, it is suggested that bivalirudin binding to thrombin is not very efficient when exosite I is occupied. Reference: Parry MA, Maraganore JM, Stone SR. Kinetic mechanism for the interaction of Hirulog with thrombin. Biochemistry 1994;33:14807–14.

Keywords: Thrombin, Bivalirudin, exosite
Overexpression of TIMP-1 in mice does not affect adipogenesis or adipose tissue development

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Matrix metalloproteinases (MMPs) may play a role in development of obesity by contributing to adipogenesis, angiogenesis and extracellular matrix degradation. To evaluate a potential functional role of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1, which inhibits most MMPs), we have overexpressed human TIMP-1 (hTIMP-1) in C57Bl/6J mice in vivo and in 3T3-F442A preadipocytes in vitro. Stable long-term overexpression of hTIMP-1 in mice was achieved by adenoviral gene transfer, yielding plasma levels exceeding 250 ng/ml at 8 weeks after injection. Mice overexpressing hTIMP-1 (n=15) and kept on high fat diet for 14 weeks had body weight (31 ± 0.8 versus 33 ± 0.5 g), subcutaneous (SC) (0.61 ± 0.08 versus 0.73 ± 0.08 g) and gonadal (GON) (1.01 ± 0.11 versus 1.24 ± 0.08 g) adipose tissue weights, and adipocyte diameters (26 ± 3 versus 28 ± 3 µm for SC, and 35 ± 4 versus 37 ± 4 µm for GON) that were somewhat, but not significantly, lower than control mice (n=16). Reduced MMP activity following hTIMP-1 gene transfer was confirmed by gelatin zymography using adipose tissue extracts and by in situ zymography using gelatin overlays on cryosections. Expression of murine TIMP-1, MMP-2 and MMP-9 mRNA in adipose tissues was not altered after hTIMP-1 gene transfer. Physical activity and food intake were comparable in both groups. Metabolic parameters, including glucose, cholesterol and triglycerides were not affected by overexpression of hTIMP-1. Histological analysis revealed that the blood vessels in adipose tissues of mice overexpressing hTIMP-1 were significantly smaller than in controls (39 ± 4.0 versus 49 ± 3.3 µm2 for SC, p < 0.001; 37 ± 3.3 versus 61 ± 3.8 µm2 for GON, p < 0.01). Similar observations were made after overexpression of hTIMP-1 in mice with lipectomy of the SC adipose tissue, kept on high fat diet for 20 weeks. No significant effects of hTIMP-1 gene transfer were observed on total body weights (41 ± 1.0 versus 41 ± 0.3 g in control mice (n= 5 or 6)), or on weights of the isolated SC (0.84 ± 0.08 versus 0.89 ± 0.05 g) or GON (2.8 ± 0.08 versus 2.8 ± 0.05 g) adipose tissues. Blood vessels were significantly smaller in hTIMP-1 overexpressing adipose tissues (14 ± 1 versus 19 ± 1 µm2 for SC, p < 0.01; 18 ± 1 versus 23 ± 2 µm2 for GON, p < 0.001). Overexpression of hTIMP-1 in 3T3-F442A preadipocytes in vitro (pIRES2 EGFP) had no effect on their subsequent differentiation into mature adipocytes, as judged from Oil Red 0 staining. Taken together, these studies reveal that overexpression of hTIMP-1 in mice reduces blood vessel size in adipose tissues without, however, a significant effect on adipogenesis or adipose tissue development. These findings thus seem to challenge the relative importance of angiogenesis in ongoing adipose tissue development.

Keywords: TIMP-1, obesity, adipogenesis
Impaired adipose tissue development in mice with inactivation of placental growth factor function

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Placental Growth Factor (PlGF) is expressed in adipose tissue, it shows functional homology with Vascular Endothelial Growth Factor (VEGF) which accounts for much of the angiogenic activity in adipose tissue, and it interacts with VEGF receptor−1 (Flt-1). To establish a functional role, we have investigated the effect of inactivation of PlGF on adipose tissue development in murine models of obesity. PlGF deficient (PlGF−/-) and wild-type (WT) mice were kept on a standard (SFD) or high fat diet (HFD) for 15 weeks. On SFD, the body weight of PlGF−/- and WT mice was comparable, whereas the combined weight of subcutaneous (SC) and gonadal (GON) adipose tissues was significantly lower in PlGF−/- mice (1.31 ± 0.17 g versus 1.95 ± 0.2 g; p= 0.02). Plasma leptin levels were significantly lower in PlGF−/- than in WT mice and correlated with adipose tissue mass in both genotypes. On HFD, PlGF−/- mice had a significantly lower body weight (53 ± 3.0 g versus 62 ± 1.5 g; p < 0.05) and significantly less total SC plus GON adipose tissue (2.90 ± 0.37 g versus 4.59 ± 0.16 g; p < 0.0001). Food intake and physical activity was comparable for both genotypes. Blood vessel density, normalized to adipocyte number, was significantly lower in SC adipose tissue of PlGF−/- mice (p= 0.001), which may contribute to the reduced fat mass. De novo adipose tissue development in NUDE mice injected with 3T3-F442A preadipocytes was significantly (14 ± 2.5 mg versus 27 ± 3.6 mg; p < 0.005) reduced by administration during 5 weeks of a monoclonal antibody (MAb) that blocks binding of PlGF to Flt-1. In contrast, this MAb did not affect adipose tissue development in WT mice kept on HFD, or in genetically obese ob/ob mice kept on SFD. Taken together, these data suggest that neutralization of PlGF function does not affect ongoing adipose tissue formation, but has the capacity to prevent or impair de novo fat pad formation. Bone marrow transplantation (BMT) from WT or PlGF−/- mice to WT or PlGF−/- recipient mice revealed significantly lower blood vessel density in PlGF−/- recipient mice, however without effect on adipose tissue growth. Following BMT, PlGF mRNA was detected only in adipose tissues of mice with BMT (WT ' WT) and (PlGF−/- ' WT), but not in BMT (WT ' PlGF−/-), indicating that recruitment of precursor cells from bone marrow to adipose tissue does not occur in this model. Thus, in murine models of diet induced obesity, inactivation of PlGF impairs de novo adipose tissue development, at least in part as a result of reduced angiogenesis.

Keywords: PlGF, obesity, angiogenesis
Elevated prothrombin level and shortened clotting times in subjects with type 2 diabetes

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A correlation has been reported between a short activated partial thromboplastin time (aPTT) and the risk of thrombosis (Corte et al, Am J Clin Path 2000, 113:123). An elevated level of prothrombin has also been associated with thrombosis. We compared aPTT and prothrombin levels in subjects with or without type 2 diabetes mellitus (DM). The subjects were the first 34 people recruited into a larger trial (n=81) on the effects of muscadine grape juice on parameters related to glycemic control and cardiovascular risk. This protocol was approved by the IRB of North Carolina State University. Subjects were initially classified as DM based on their report of having been diagnosed by a physician. They were subsequently reclassified based on fasting glucose, insulin and glycated hemoglobin (HbA1C) levels. Self-classification into DM or control groups was verified for all except one subject who was excluded from the analysis. Her fasting glucose and HbA1C were within the reference range, but her fasting insulin level was elevated suggestive of insulin resistance. DM subjects had higher prothrombin levels than gender-matched controls, and male controls had higher prothrombin than female controls (all p<0.05). DM subjects had a significantly shorter aPTT than controls. Male controls had a shorter mean aPTT than females, but the difference did not reach statistical significance. PT times were also shorter for DM than control subjects. The difference was statistically significant for females (p=0.01), but just missed significance for males (p=0.06). In the DM groups there was a significant inverse correlation between the prothrombin level and the PT and aPTT. This suggests that the elevated prothrombin level in DM is an important contributor to the shortened clotting times. There was no significant difference in fibrinogen or plasminogen activator inhibitor-1 (PAI-1) levels between the groups. In summary, shorter clotting times and higher prothrombin are found in subjects with a higher risk of atherothrombotic disease: type 2 diabetics and males. This suggests that the prothrombin level may be influenced by by hormonal factors and deserves further study as a possible contributor to cardiovascular risk.

Keywords: thrombosis, diabetes, coagulation

* selected for oral presentation  
† not represented at ISFP Congress
Cathepsin S Expression in Mouse Obesity Models

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Cathepsin S (CatS) is an elastolytic cysteine protease and has been implicated in the development of atherosclerosis in both animal models and in human. Recent studies showed that CatS is a circulating biomarker in obese patients, and CatS blocks HDL-induced cholesterol efflux from macrophage foam cells. We studied the CatS expression in mouse obesity models. Comparing to the lean control mice, ob/ob mice have a 4-fold increase (p=0.0001) of CatS expression by real-time PCR. A 16-hour fasting before tissue harvesting did not significantly affect the expression of CatS, while it significantly reduced CD11b expression, suggesting that the CatS expression was not acutely regulated by blood glucose level. In a diet-induced obesity mouse model, there was a time-dependent increase of CatS expression. The CatS expression was 7-fold higher at 24-weeks than that at 3-weeks (p=0.0004). Immunohistological studies showed that CatS antigen was positive in both adipocyte and macrophage in the ob/ob white adipose tissue (WAT) and in the extra-cellular matrix. Significant expression of CatS in adipocytes isolated from WAT depleted stroma vascular fraction was confirmed by real-time PCR. Furthermore, cysteine protease activity was detected using a fluorogenic substrate in the WAT and was inhibited by a CatS inhibitor. In an in vitro study, time- and dose-dependent degradation of apoA-I and HDL by CatS was observed. Our data demonstrate that CatS was produced by adipocyte and increased in obesity animal models indicating that CatS may be a molecular link between obesity and dyslipidemia in metabolic syndrome. In addition to its proinflammation and matrix degradation activities, CatS inactivate HDL and therefore may increase the risk of cardiovascular disease in obese subjects.

**Keywords:** Obesity, Cathepsin S, dyslipidemia
Keratinocyte-derived chemokine in adipocyte biology and obesity: Potential role in adipose inflammation and glucose homeostasis.

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The obese adipose tissue is associated with chronic inflammation, a condition that may contribute to obesity-related cardiovascular and metabolic disorders including accelerated atherosclerosis, insulin resistance and type 2 diabetes. Adipocytes secrete a variety of cytokines/chemokines including tumor necrosis factor alpha (TNF-alpha), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and many more, and it has been suggested that these "adipokines" may play a role in the pathogenesis of some of these health complications. In this study, we characterized the expression, regulation, and functional significance of keratinocyte-derived chemokine (KC), the mouse ortholog of human IL-8, in adipose tissue biology and pathology. 3T3-L1 pre-adipocytes in culture expressed high levels of KC, which decreased significantly during differentiation to mature adipocytes. This was also confirmed in an in vivo model of adipogenesis. In the in vivo model of adipose tissue development where F442A pre-adipocytes were implanted into athymic Balb/c nude mice, KC mRNA levels were high on days 1–2 post-implantation; this expression decreased rapidly during the course of adipogenesis and adipose tissue development. In this vivo model, the initial high levels of KC expression preceded a dramatic influx of macrophages from the host into the developing fat pad, suggesting a potential role for this chemokine in macrophage recruitment into the adipose tissue. KC expression was significantly increased in the adipose tissue and plasma of genetically obese (ob/ob, db/db) mice and, our data suggest that these increases may be mediated by the elevated insulin, leptin and TNF-alpha associated with obesity. Finally, we present data that suggest a role for KC in glucose homeostasis and insulin resistance, and in the regulation of several cardiovascular and metabolic risk genes in adipocytes (i.e., MCP-1, TNF-alpha, IL-6, leptin and KC itself.) These studies are consistent with the hypothesis that adipose synthesis of KC may potentially contribute to the chronic systemic inflammation, insulin resistance, and accelerated atherosclerosis associated with obesity.

Keywords: obesity, inflammation, adipose tissue
The 4G Genotype Plasminogen Activator Inhibitor - I (PAI-I) is associated with Deep Vein Thrombosis in Saudis.

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An impaired fibrinolytic activity due mostly to increased levels of the Plasminogen Activator Inhibitor -I (PAI-I) is commonly observed in patients with thrombotic disease including those with ischeamic heart disease as well as in patients with variables defining the Insulin Resistance Syndrome. There is conflicting data to its association with venous thrombosis. PAI-I levels correlates with several polymorphism particularly the one at the promotor region 675 bp known as the 4G/5G polymorphism. PAI-I antigen activity is highest in individuals homozygous to 5G/5G, and intermediate in those with the 4G/5G and lowest in those with the 4G/4G polymorphism. The prevalence of the 4G/5G polymorphism in the Saudi Population is not known. We aimed to study the prevalence and its possible association with deep vein thrombosis (DVT) in the Saudi population. Blood from consenting normal controls and patients with DVT was drawn and tested for the 4G/5G polymorphism using established methods. The clinical diagnosis of DVT was always confirmed by radiological investigations. We compared the prevalence of the 4G and 5G alleles in normal and patient populations. We also looked at possible interaction of the 4G allele with factor V Leiden (FVL), prothrombin (PT) 20210 G>A mutant gene, the 5–10 methylenetetrahydrofolate reductase (MTHFR) 677C >T, and age. Our results show that in normal Saudis the prevalence of the 4G/5G polymorphism was 37.8% for the 5G/5G, 52.5% for the 4G/5G and 9.6% for the 4G/4G. Patients with DVT were homozygous for 5G/5G in 27% heterozygous 4G/5G in 60% and homozygous for 4G/4G in 12.9%. The prevalence of the 4G allele is patients with DVT were much higher in patients than in the control population (P<0001). The association of FVL plus the 4G allele conferred a significantly higher risk of DVT; the association of age, FVL and the 4G allele conferred an even higher risk. The association of the PT 20210 G>A and the 4G allele conferred a higher risk for DVT but did not reach a statistical significance. However, the combination of age, PT 20210 G>A and the 4G allele did confer a significantly high risk of DVT. MTHFR 677 C>T did not add to the risk of DVT in patients with 4G allele. In normal Saudi population the prevalence of the 4G/5G polymorphism is roughly similar to what is found in most other population. However, the prevalence of the 5G/5G polymorphism is at the highest range and that of the 4G/4G polymorphism is at the lowest range. The prevalence of the 4G allele is significantly higher in patients with DVT than in normal. The combination of FVL Age and the 4G allele confer a significantly increasing risk of DVT. The combination of the PT 20210 G>A and the 4G allele confer a statistically non-significant increase in risk. However, the combination of Age, PT 20210 G>A and the 4G alleles does confer a significant increase in the risk for DVT. There does not appear to be an augmentation of risk with the combination of MTHFR and the 4G allele.

Keywords: Plasminogen Activator Inhibito, Deep vein thrombosis
Hepatocyte Growth Factor Induces Plasminogen Activator Inhibitor Type 1 Gene Expression in Human Hepatocytes in an E Box-Dependent Manner: A Novel Link Between Hypofibrinolysis and Metabolic Syndrome

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Angiogenesis-dependent wound healing is initiated by hemostasis. In the regenerating tissue plasminogen activator inhibitor (PAI)-1, the major physiologic inhibitor of fibrinolysis, preserves extracellular matrix (ECM) scaffold permissive for cell migration. Thus, PAI-1 has a critical role in angiogenesis and subsequent wound healing. Patients with acute coronary syndrome exhibit increased serum levels of hepatocyte growth factor (HGF), which stimulates angiogenesis. We have previously shown that plasma levels of PAI-1 increase in thrombotic states. HGF stimulates hepatic PAI-1 expression in vitro, but little is known how this pathway is modulated. Because liver is one of the major sources of circulating PAI-1, the effects of HGF on hepatic PAI-1 production were determined using HepG2 cells, a highly differentiated human hepatoma derived cell line with many characteristics of human liver cells. HGF increased PAI-1 accumulation in the conditioned media after 12 hours in a dose-dependent manner (2.3 ± 1.3 fold over control at 0.5 ng/ml, 3.5 ± 1.3 fold at 5 ng/ml, 7.9 ± 2.7 fold at 50 ng/ml and 11.1 ± 5.6 fold at 100 ng/ml, Western blot, n = 3). HGF induced PAI-1 mRNA expression (1.7 ± 0.4 fold over control at 0.5 ng/ml and 3.3 ± 0.9 fold at 50 ng/ml, n = 3, Northern blot). Increase of PAI-1 mRNA by HGF was attenuated by U0126, a specific inhibitor of mitogen-activated protein kinase kinase, and genistein, an inhibitor of tyrosine kinase. In contrast, GF109203X, an inhibitor of the protein kinase C pathway, and LY294002, an inhibitor of phosphatidylinositol 3-kinase, exerted no effects. Intravenous injection of HGF (100 microg/kg) increased hepatic PAI-1 mRNA expression in mice in vivo (1.4 fold over control, real-time PCR), exhibiting the physiological relevance of HGF effects in vitro. Transient transfection assay of the human PAI-1 promoter–luciferase construct demonstrated that HGF increased PAI-1 promoter (~829 to +36bp region) activity by 2.0 ± 0.3 fold (n = 5), and subsequent deletion and mutation analyses uncovered a functional E box motif (5’-CACATG-3’) at positions -158 to -153bp in the PAI-1 promoter. Electrophoretic mobility shift assay demonstrated that this E box motif binds heterodimers of upstream stimulatory factor (USF)-1 and -2. As USFs are known to control the hepatic production of metabolic enzymes such as fatty acid synthetase, HGF mediated PAI-1 production may provide a novel link between thrombosis and metabolic syndrome. Targeting HGF signaling pathway may modulate the thrombotic risk in patients with metabolic syndrome.

Keywords: PAI-1, gene regulation, metabolic syndrome
Murine plasminogen activator inhibitor-1 (PAI-1) does not affect adipogenesis or adipocyte differentiation

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Development of obesity is associated with extensive remodeling of adipose tissue. The plasminogen/plasmin (fibrinolytic) system, which contributes to tissue remodeling by degradation of extracellular matrix, plays a role in the regulation of adipose tissue growth. The functional role of PAI-1, a main inhibitor of the system, in obesity remains, however, controversial. In this study, the role of PAI-1 in adipogenesis and adipocyte differentiation was investigated both in vitro and in vivo murine model systems. The rate and extent of differentiation of cultured primary embryonic fibroblasts, derived from wild-type (WT) or PAI-1 deficient (PAI-1−/−) mice, into mature adipocytes was not significantly different, as monitored by Oil Red O staining. Semi-quantitative RT–PCR confirmed comparable expression of several adipogenic markers, including aP2, Pref1, PPARgamma and GPDH. Differentiation of 3T3–F442A murine preadipocytes was not affected by treatment with a PAI-1 neutralizing monoclonal antibody (mAb H4B3) (60 to 70% differentiation at 10 days after induction). This finding was supported by comparable expression levels of adipogenic markers. Differentiation of 3T3–F442A preadipocytes stably transfected with the pIRES2–EGFP–PAI-1 vector to overexpress murine PAI-1 was similar to that of preadipocytes transfected with the empty pIRES2–EGFP vector (75 to 80% differentiation at 10 days after induction). Again, there was no difference in the expression of aP2, Pref1, PPARgamma or GPDH. Subcutaneous injection of 3T3–F442A preadipocytes in the back of NUDE mice resulted in comparable de novo formation of fat pads in mice treated for four weeks with mAb H4B3 or with the control mAb 1C8, and kept on high fat diet (HFD). Plasma PAI-1 antigen levels were higher in mice treated with mAb H4B3 (8.8 ± 0.5 ng/ml versus 3.3 ± 0.2 ng/ml for mAb 1C8; p < 0.01). Weight gain during the experiment, as well as the weights of the de novo formed fat pads and of the subcutaneous (SC) and gonadal (GON) adipose tissues were comparable in both groups. Histological analysis revealed a significantly lower adipocyte size in de novo fat pads of mAb H4B3 treated mice (1120 ± 98 µm² versus 1440 ± 93 µm² for mAb 1C8; p = 0.03), whereas adipocyte density as well as blood vessel size and density were not significantly different for both groups. Injection of Matrigel together with basic fibroblast growth factor resulted in de novo formation of fat pads in both WT and PAI-1−/− mice kept on HFD for 4 weeks. Plasma PAI-1 levels were 5.4 ± 2.3 ng/ml at the time of sacrifice in WT mice and were undetectable in PAI-1−/− mice. Weight gain during the experimental period, as well as the weight of the isolated Matrigel plugs and of SC adipose tissue, were comparable for both genotypes. No significant differences in adipocyte size or density were observed between WT and PAI-1−/− mice whereas the blood vessel size was higher in PAI-1−/− SC adipose tissues (22 ± 2 µm² versus 15 ± 3 µm² for WT; p= 0.048). Taken together, these studies indicate that murine PAI-1 does not play an important functional role in adipogenesis or adipocyte differentiation.

Keywords: PAI-1, obesity, adipogenesis
PLASMINOGEN ACTIVATOR INHIBITOR-1 PLASMA LEVELS ARE ASSOCIATED WITH CORONARY INSTENT RESTENOSIS OF DRUG ELUTING STENTS

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BACKGROUND. Percutaneous coronary intervention (PCI) represents the most important treatment modality of coronary artery stenosis today. Although with the introduction of drug eluting stents (DES) in-stent restenosis (ISR) could be reduced dramatically, it still plays a significant role in the long-term outcome after PCI. The fibrinolytic system is believed to be of pathophysiological relevance in the development of ISR.

METHODS. We studied 75 patients (median age 64, 56 male). Blood samples were taken directly before and 24 hours after PCI with DES implantation. Restenosis was evaluated at 6 to 8 months by coronary angiography.

RESULTS. During the follow up period, 2 patients (2.7%) died of cardiovascular causes and ISR was detected in 12 patients (16%). At baseline, patients with ISR at follow-up angiography showed significantly lower plasma levels of PAI-1 active antigen compared to patients without ISR (11.7±8.1 vs. 22.8±18.8; p<0.05). Patients with PAI-1 active antigen in the lowest tertile showed a 9.5-fold risk of ISR compared to patients in the third tertile (p<0.05). The PCI-induced change of PAI-1 active antigen was significantly higher in patients with ISR as compared to patients without ISR (+5.6±8.0 ng/mL vs −3.2±12.1 ng/mL; p<0.05). Multiple regression analysis revealed that late lumen loss was associated with the PCI-induced change of PAI-1 active antigen and inversely correlated with PAI-1 active antigen before PCI independent from stent diameter, stent length, type of stent, number of stents, stented vessel as well as presence of diabetes.

CONCLUSION. The occurrence of ISR showed a significant correlation with baseline plasma levels of PAI-1 active antigen before PCI and the change of PAI-1 active antigen due to PCI. As PAI-1 may play a role in the pathogenesis of ISR, determination of PAI-1 plasma levels might be helpful in the identification of patients with high risk for development of IRS after DES implantation.

Keywords: STENT, RESTENOSE, PAI-1
Elucidation of the molecular interaction mechanism between PAI-1 and a PAI-1 inhibiting antibody fragment Fab-55F4C12

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Introduction: Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (serpin) superfamily and is the principal inhibitor of the plasminogen activators tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in vivo. In healthy individuals, PAI-1 is found at low levels in the plasma, but is elevated significantly in a number of diseases, including atherosclerosis and deep vein thrombosis. Objective: Elucidation of the molecular interaction mechanism between PAI-1 and a PAI-1 inhibiting antibody fragment Fab-55F4C12.

Methods and results: Fab-55F4C12 was generated by papain digestion of MA-55F4C12, followed by protein A and gel filtration purification. The purified Fab-55F4C12 was concentrated to a concentration of 10 mg/ml. Different crystallisation screens were tried, and initial crystal clusters were obtained in condition 19 of Structure Screen 1 of Molecular Dimensions (0.2M Zinc acetate dehydrate, 0.1M Na Cacodylate pH 6.5 and 18% w/v PEG 8000). Small needles (0.2 x 0.1 x 0.1 mm) were obtained after intensive optimisation of the crystallisation conditions. The crystal structure of Fab-55F4C12 was determined by X-ray crystallography at cryogenic temperature. The data set was collected at DESY (Hamburg, Germany) to a resolution of 2.7 Å. Data processing was done using MOSFLM and SCALA. The space group was assigned to be P21212 with unit-cell parameters a = 52.04 Å, b = 98.66 Å, c = 191.68 Å, with two molecules in the asymmetric unit. The data set is 99.72 % complete. Initial phases were obtained with molecular replacement. The structure was refined using Coot and Refmac5.

Crystallisation of the Fab-55F4C12 / PAI-1 complex has been unsuccessful so far. Therefore, the complex is being modelled through docking of the crystal structures of the two subunits, i.e. Fab-55F4C12 and PAI-1, using the rigid-body docking programs DOT and ZDOC. Resulting models are filtered based on the available biochemical information (e.g. binding regions) and will be validated with small-angle X-ray scattering (SAXS). Potential interactions between the two subunits will be deduced from the models and compared with epitope information gathered from mutagenesis studies.

Conclusions: Characterization of the complex of Fab-55F4C12 with PAI-1 may provide valuable information on the molecular interactions between the Fab-fragment and PAI-1, leading to a better understanding of the mechanism of inhibition. The elucidation of the binding site of inhibitory monoclonal antibodies may contribute to the rational design of PAI-1 modulating therapeutics.

Keywords: PAI-1, crystallization, modeling

* selected for oral presentation
† not represented at ISFP Congress
The epitope of MA-31C9, a non-inhibitory anti-PAI-1 antibody, overlaps with the epitope of a previously identified inhibitory antibody

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Introduction: Plasminogen Activator Inhibitor 1 (PAI–1) is a member of the serine protease inhibitor (serpin) superfamily and is the most important physiological inhibitor of plasminogen activators in vivo. In healthy individuals, PAI–1 is found at low levels in plasma, but is significantly elevated in a number of diseases, including atherosclerosis, deep vein thrombosis, and non-insulin dependent diabetes mellitus. PAI–1 antigen levels in human and primate plasma are determined using the MA–31C9/MA–55F4C12–HRP ELISA. The epitope of MA–55F4C12 was previously mapped to E128V129E130R131K154. MA–31C9 is a non-inhibitory monoclonal antibody raised against human PAI–1. It is often used as a control antibody in studies on the evaluation of PAI–1 inhibiting antibodies. Objective: To elucidate the epitope of MA–31C9, a non–inhibitory anti–PAI–1 antibody.

Methods and Results: Wild–type human PAI–1 has a high affinity for MA–31C9 (i.e. KA = 3.4 ± 1.0 x 10e9 1/M using surface plasmon resonance (SPR)) whereas rat, murine and porcine PAI–1 do not bind to MA–31C9 (KA < 10e6 1/M). Moreover, a PAI–1 chimera harbouring residues 1–26 of rat PAI–1 and 27–379 of human PAI–1 does not bind to MA–31C9 (KA < 10e6 1/M) whereas a PAI–1 chimera harbouring residues 1–81 of human PAI–1 and 82–379 of rat PAI–1 does bind to MA–31C9 (KA = 1.1 ± 0.03 x 10e9 1/M). Based on these findings, we hypothesized that the epitope of MA–31C9 must reside in α-helix A. Sequence alignment of the first 26 residues of human PAI–1 versus rat PAI–1 revealed 5 residues that are charged, surface exposed and that differ between human and rat PAI–1 (i.e. H2, H3, H10, D14, R18 in human PAI–1). These 5 residues were replaced by alanine. Following expression in E.coli, the crude lysates of these single PAI–1 mutants were subjected to SPR analysis. These experiments revealed a decreased binding of the PAI–1–H3A and PAI–1–H10A variants for MA–31C9. These PAI–1 variants were purified and subsequent affinity measurements revealed KA values of 4.3 ± 1.4 x 10e7 1/M and 4.3 ± 4.1 x 10e7 1/M for PAI–1–H3A and PAI–1–H10A, respectively. Based on in silico analysis of these positions, the contribution of Y7 is hypothesized. A PAI–1–Y7A variant was constructed, expressed and purified. SPR analysis revealed a minor but significant contribution of this residue (KA = 5.9 ± 0.9 x 10e8 1/M) to the epitope of MA–31C9.

Conclusions: We identified three residues (i.e. H3, Y7, H10) that contribute to the epitope of MA–31C9. These residues are located in α-helix A, a region previously identified as the binding region of MA–159M12, a rat PAI–1 antibody that accelerates the active to latent transition of rat PAI–1. These data indicate that either subtle structural differences between human and rat PAI–1 are responsible for this apparent discrepancy or that subtle differences in the binding orientation of the monoclonal antibody may play a role in their different functional effects.

Keywords: PAI–1, epitope, monoclonal antibody

* selected for oral presentation 163
† not represented at ISFP Congress
Analysis of blood platelet plasminogen activator inhibitor under treatment by streptokinase

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Much attention is paid to plasminogen activators and their inhibitors because of their important role in fibrinolysis and in a wide variety of biological function including tissue remodeling, cell migration, carcinogenesis and neoplasia. Fibrinolysis, which keeps the vascular compartment patent, is regulated by the activities of both tissue type plasminogen activator and its inhibitor (PAI-1). Streptokinase (Sk) is bacterial protein widely used a thrombolytic agent in the treatment of coronary thrombosis. Our previous investigation has manifested the PAI-1 activity changes in patients treated by streptokinase. As 90% of PAI-1 accumulates in alpha-granules of platelets the aim of our investigation is to study the features of platelets’ plasminogen activator inhibitor under treatment by streptokinase. Blood was drawn from rabbits into 10% final volume of 3.8 % sodium citrate. Platelet–rich plasma (PRP) was prepared by centrifugation of whole blood at 150g at 10 minutes. For some experiments, PRP was depleted of plasminogen by passing it over a column of lysine Sepharose 4B (Amersham Biosciences). The characteristics of platelet PAI-1 after addition of streptokinase were determined with used of ELISA and Western blotting. Platelets were treated with streptokinase and analyzed by flow cytometer (FACScan flow cytometer, Becton Dickinson). The studying of PAI-1 under treatment by streptokinase was carried out in vivo with used of rabbits. In the presence of streptokinase (5, 50, 200 U/ml) added to untreated PRP, the PAI-1 quantity was significant increased. High concentrations of streptokinase 200 U/ml cause the elevation of PAI-1 quantity in 1,6 times from platelets. We characterized the PA–inhibitor from platelets after treated by streptokinase with used of Western blotting. It was shown the presence of PAI-1 in free (55 kDa) and complexes forms with MW 70 kDa and 100 kDa. After intravenously injection of streptokinase in 1 hour PAI-1 quantity was increased in 2 times and mounted to 16,7 ng/ml compared to normal (8,9 ng/ml). On the next stages of investigation (3, 7 days) this parameters tend to norm. Streptokinase causes platelet activation and aggregation in untreated PRP immediately after addition. The effect of streptokinase was eliminated after the depletion of plasminogen from PRP but simultaneous influence of streptokinase and Sk–antibodies cause platelet activation. On the basis of the received data we concluded that intravenously injection of streptokinase can cause PA–inhibitor secretion from ?–granules of platelets due to platelets activation. That can lead to occurrence of thrombotic complications risk. PAI–1 is secreted in free and complex forms after the streptokinase addition. Streptokinase can influence on platelets also without plasmin participates. Understanding of these mechanisms is crucial for a proper selection of thrombolytic therapies and provides the rational for development of novel therapeutic options.

Keywords: PAI-1, streptokinase, platelets
Plasminogen activator inhibitor 1 forms a stable non-acyl enzyme inhibitor complex with tissue plasminogen activator

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Aim Members of the superfamily of serine protease inhibitors (SERPINS) share similar tertiary structure and interact with the active site of target serine proteases at their reactive site peptide bond (P1–P1’), located near the carboxyl terminus. Although the cleavage of the P1–P1’ peptide bond and irreversible acyl–enzyme inhibitor complex formation are essential for inhibition, the intermediate sequential events that occur during complex formation have not been fully elucidated. Employing tissue plasminogen activator (tPA), a serine protease possessing inherent activity in its single chain form (sctPA), and plasminogen activator inhibitor type 1 (PAI–1), the molecular masses of the complexes formed were analyzed.

Results After incubation of sctPA and recombinant PAI–1 at a 1:1 molar ratio for 30 min, two distinct forms of tPA/PAI–1 complexes were detected by SDS–PAGE and MALDI–TOF mass spectrometry. The high molecular weight form corresponded to 107,929 Da, reacted with a monoclonal antibody raised against the C-terminal cleavage peptide of PAI–1 (P1Met–P17Arg), whereas the low molecular weight (104,367 Da) form did not. MALI–TOF MS revealed that the mixture contained another peptide peak of 3,808 Da, a similar value to the difference in the molecular mass between the two tPA/PAI–1 complex forms, suggesting that the low molecular weight form lacks the C–terminal peptide (calculated mass, 3803.6 Da) of PAI–1 after cleavage at the P1–P1’ bond by tPA. Treatment of the complex with 0.1 % SDS/1.0 % Triton X–100 increased the amounts of the low molecular weight–form, as well as its dissociated forms of free tPA and cleaved forms of PAI–1. On fibrin autography, both free tPA and the high molecular weight form of the complex demonstrated tPA activity, whereas the low molecular weight complex form did not.

Conclusion PAI–1 appeared to form a stable non–acyl enzyme inhibitor complex with sctPA.

Keywords: SERPIN, PAI–1, tPA
S-35225 decreases plasma PAI-1 activity both in vitro and in vivo

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The active form of PAI-1 is the major inhibitor of plasminogen activators and elevated PAI-1 levels are associated with thrombosis and vascular diseases. Recent research efforts have resulted in the identification of several synthetic compounds capable of reducing PAI-1 activity in different in vitro and in vivo assays. In the present study, we describe the inhibitory effects of the benzothiophene derivative PAI-1 inhibitor S-35225 and compare its activity to that of two other PAI-1 inhibitors Tiplaxtinin and WAY-140312. In a direct in vitro assay using purified urokinase and a chromogenic substrate, S-35225 inhibits human recombinant PAI-1 activity with an IC50 value of 44 +/- 0.9 µM, a value similar to that of Tiplaxtinin (34 +/- 7 µM) and WAY-140312 (39 +/- 1 µM). In a clot lysis assay using human purified proteins however, S-35225 has a significantly lower IC50 value than Tiplaxtinin and WAY-140312 (0.6 +/- 0.3 versus 22 +/- 5 and 16 +/- 2 µM, respectively). Moreover, when tested in a plasma milieu using a tPA capture assay, neither WAY-140312 nor Tiplaxtinin reached 50% inhibition at the highest concentration tested (1 mM) while S-35225 has an IC50 value of 194 +/- 30 µM against rat recombinant active PAI-1 and 260 +/- 41 µM against human recombinant active PAI-1. In vivo in the rat, only S-35225 reduced active PAI-1 levels in the blood (maximum inhibition of 76 +/- 5% at 10 mg/kg and 53 +/- 5% at 3 mg/kg, intravenously) while we were unable to detect inhibition of active PAI-1 with either Tiplaxtinin or WAY-140312 at the highest dose tested (10 mg/kg, intravenously). In conclusion, the benzothiophene derivative S-35225 is a functional inhibitor of active PAI-1 in vitro which preserves its activity in plasma both in vitro and in vivo. The fact that S-35225 inhibits PAI-1 activity in human plasma may be helpful to evaluate its pharmacodynamic properties during future development.

Keywords: PAI-1 inhibitor, PAI-1 activity, thrombolytic therapy
Efficacy of PAI-749, an orally active PAI-1 inhibitor, in dog and rat models of arterial and venous thrombosis

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Plasminogen activator inhibitor–1 (PAI–1) is the major physiological inhibitor of tissue–type plasminogen activator (t-PA) and is elevated in diseases associated with a prothrombotic state. In this study, we characterize the in vivo antithrombotic efficacy of PAI–749, a small molecule orally–active inhibitor of active PAI–1. In the ferric chloride rat model of arterial injury, the time to carotid artery thrombosis was significantly delayed with PAI–749 oral doses of 0.3 mg/kg (41.2 plus/minus 9.0 min), 1.0 mg/kg (31.2 plus/minus 10.3 min) and 3 mg/kg (52.7 plus/minus 7.3 min) compared to vehicle–treated controls (11.8 plus/minus 0.4 min). Complete cessation of blood flow was observed in 100% of the vehicle–treated controls, but PAI–749 prevented occlusion in 43% and 83% of rats treated with 1 mg/kg and 3 mg/kg, respectively. PAI–749 was also efficacious in the rat venous thrombosis model, significantly reducing ferric chloride–induced thrombus weight in the vena cava at 0.3 (8.6 plus/minus 1.6 mg) and 1 mg/kg (8.0 plus/minus 0.2 mg) compared to controls (16.5 plus/minus 2.6 mg). PAI–749 had no effect on tail bleeding time or thrombin clotting time in the rat. PAI–749 was also tested for in vivo efficacy in a canine model of electrolytic injury–induced thrombosis. PAI–749 significantly prolonged time to coronary occlusion at 3.0 mg/kg; spontaneous reperfusion was observed in PAI–749 treated dogs (80% of dogs treated at 1.0 mg/kg and 67% of dogs treated at 3.0 mg/kg). Heart rate, mean arterial blood pressure, tongue template bleeding time, APTT, PT and platelet aggregation were not changed by the oral administration of PAI–749 to dogs. Canine ex vivo platelet aggregation was also not affected by PAI–749 treatment. The pharmacokinetics and bioavailability of PAI–749 was evaluated in male rats after a 3 mg/kg IV bolus or 15 mg/kg oral gavage. PAI–749 had an oral bioavailability of 45% and a half–life of 4.5 hours after oral dosing in the rat. In the dog, PAI–749 had a 37% oral bioavailability and 7.9 hour half–life. These results demonstrate that oral administration of PAI–749 prior to vascular injury leads to sustained blood flow, reduced thrombus size, yet preservation of normal hemostasis.

Keywords: PAI–1, thrombolysis, thrombosis
Effect of plasminogen activator inhibitor-1 on growth of mouse melanoma cells

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Plasminogen activators (PAs) such as tissue-type PA (t-PA) and urokinase-type PA (u-PA), activate plasminogen to plasmin. Plasmin directly breaks down the extracellular matrix or indirectly it through activation of pro–metalloproteinases to active form of metalloproteinases. Therefore, the activation of plasminogen by PA has been thought to play an important role in tumor growth. Since PA inhibitor–1 (PAI–1) inhibits PA activity, PAI–1 has thought to be a suppressor for tumor growth. However, various clinical studies showed that the high PAI–1 level demonstrated a poor prognosis for the survival of cancer patients. In the present study, the effects of PAI–1 on tumor growth were investigated. Mouse melanoma (B16) cells were injected into the subcutaneous layers of wild-type and PAI–1 deficient mice. After four weeks, grown tumors were removed and their weights were measured. The weights of tumor obtained from wild-type mice were significantly greater than those from PAI–1 deficient mice.

The activity of caspase 3 was detected by western blot for active caspase 3. The activity of caspase 3 in tumor obtained from PAI–1 deficient mice was higher than that from wild-type mice. To investigate whether the presence of PAI–1 in host animal influences on the proliferation and the apoptosis of melanoma cells, fibroblast was prepared from wild-type mice or PAI–1 deficient mice. And mouse melanoma cells were cultured with wild-type fibroblast or with PAI–1 deficient fibroblast in double chamber system. Namely, each fibroblast was cultured in upper chamber and mouse melanoma cell was cultured in lower chamber. In the presence of wild–type fibroblast the time dependent increase of mouse melanoma cell was greater than that in the presence of PAI–1 deficient fibroblast. The activity of caspase 3 in mouse melanoma cell cultured with PAI–1 deficient fibroblast was significantly higher than that with wild–type fibroblast. The PA activity in the medium was studied by using fibrin zymography. The PA activity of the medium in which mouse melanoma cells were cultured with PAI–1 deficient fibroblast, was higher than that with wild–type deficient mice. To investigate the stimulation of growth, total and the phosphorylated ERK1 and ERK2 levels were measured by western blot. The phosphorylated ERK1 and ERK2 levels in mouse melanoma cells cultured with wild–type fibroblast were compared with those cultured with PAI–1 deficient fibroblast. No significant differences were observed between in the presence of wild–type fibroblast and PAI–1 deficient fibroblast. Thus it was clarified that the deficiency of PAI–1 in host mouse or fibroblast did not influence on the stimulation of growth in transplanted or co–cultured melanoma cells, but induced apoptosis of them.

Keywords: PAI–1, apoptosis, melanoma
Mechanistic Studies on the Inactivation of Plasminogen Activator Inhibitor (PAI-1) by PAI-749, a Small Molecule PAI-1 Antagonist


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PAI-749 is a potent and selective small molecule antagonist of plasminogen activator inhibitor 1 (PAI-1) that preserves tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activities in the presence of PAI-1 (EC50 values are 154 and 86 nM, respectively). Addition of PAI-749 to fluorophore-tagged PAI-1 (PAI-NBD119) quenches the fluorescence (Fl) signal in a concentration dependent manner; the apparent Kd ~ 254 nM is similar to the EC50 (~ 140 nM) for inactivation of PAI-NBD119 using tPA. Analogs of PAI-749 display the same rank order of potency for neutralization of PAI-1 inhibitory activity and perturbation of the PAI-NBD119 Fl signal. These data establish that PAI-749 binds directly to PAI-1, and that the binding event altering the Fl signal of PAI-NBD119 is linked to PAI-1 inactivation. Treatment of PAI-1 with PAI-749 and subsequent PAI-749 sequestration by Tween-80 detergent micelles spared PAI-1 from inactivation; hence, PAI-749 does not irreversibly inactivate PAI-1, a known metastable protein. Addition of tPA to preformed complex between another fluorophore-tagged PAI-1 (PAI-NBDP1) and PAI-749 perturbs the Fl signal but does not yield the canonical SDS-stable complex. This result points to the existence of a reversible ternary complex between PAI-1, PAI-749 and tPA. PAI-1 in this putative ternary complex does not “lock-in” to the active site of the PA as judged by results from p-aminobenzamidine displacement studies and lack of formation of the SDS-stable PA/PAI-1 complex. PAI-749 does not appreciably turn PAI-1 into a substrate for tPA; however, PAI-749 promotes plasmin-mediated degradation of active PAI-1 (but not latent PAI-1). In conclusion, the antithrombotic efficacy of PAI-749 (J. Hennan et al, unpublished) arises from the ability of PAI-749 to bind directly to PAI-1, interfere with formation of the acyl-enzyme intermediate and block formation of the SDS-stable tPA/PAI-1 complex. Plasmin-mediated PAI-1 degradation in the presence of PAI-749 is an indirect mechanism by which PAI-749 may additionally neutralize PAI-1 at sites of active fibrinolysis thus contributing to the antithrombotic efficacy of this compound.

Keywords: PAI-1 antagonist, profibrinolytic, PAI-1
Rational design of cysteine mutants of PAI-1 with very long half-lives

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Proteolytic activity initiated by uPA is commonly recognized as a critical factor in angiogenesis and metastasis. Many cancers overexpress uPA and the reduction of proteolytic activity has been proposed as a cancer treatment option. Indeed, uPA inhibitors have been shown to reduce angiogenesis and tumor growth. Thus, we want to identify novel inhibitors of uPA suitable for cancer treatment. We have chosen PAI-1, which inhibits the urokinase plasminogen activator. However, PAI-1 is not a stable molecule and converts itself into the latent form with a half-life in the range of $t_{1/2} \approx 2$ hours. Based on the known structure of active PAI-1, we have identified amino acids that can be substituted with a cysteine residue to produce disulfide bridges linking the top and bottom parts of strands A3 and A5 as well as sites within the helix D region in hopes of preventing a conversion into the latent PAI-1. We have created a total of seven cysteine mutants via point mutation (two to six point mutations) generating possible sites for a disulfide bridge formation at the top and bottom parts of A3 and A5, within the helix D region, or by a combination thereof. Desired mutations were introduced by PCR using appropriate primers. The mutant forms of PAI-1 containing the chitin binding intein tag were then purified using affinity chromatography wherein the intein tag is cleaved leaving the mutant PAI-1 protein. Cysteine mutations resulted in proteins with an extended half-life of PAI-1 from 2 to over 700 hours depending on the mutant. Novel PAI-1s were fully functional against uPA and showed activity in the in vitro model of angiogenesis e.g. inhibition of sprout formation. The mutant with the longest half-life (one that produced a disulfide bridge linking the top part of strands A3 and A5) was chosen for further study. VLHL PAI-1 expressed in the E. Coli vector produced a modest yield of 1 mg purified protein from 1L of cell culture. Thus, we expressed it in the baculovirus vector, with an 6His purification tag that produced \( \sim 18 \) mg of PAI-1/1L. Two different forms were made: fully active VLHL PAI-1 and VLHLNS PAI-1 with an Arg369?Ala mutation in the P1 position, which will be used in future anticancer study as a negative control. VLHLNS PAI-1 mutation (Arg369?Ala) was introduced by PCR and gene was transferred into the baculovirus vector in the same way as VLHL PAI-1. We assume that the VLHLNS construct will remain in an active conformation as VLHL PAI-1 does, but will not have any inhibitory activity toward uPA. Our study suggests that both proteins are in an active conformation, however they convert into the latent form after treatment with reducing agents, which break the disulfide bridge. Fully active VLHL PAI-1 inhibits uPA as demonstrated by chromogenic assay (SPEC-TROZYME®) and forms the uPA/PAI-1 complex as shown on PAGE gel while VLHLNS PAI-1 does not. We believe that PAI-1s with extended half-lives are therapeutically desired in cancer treatment and Cys mutated PAI-1s could launch a new class of novel anti-cancer agents.

Keywords: PAI-1, cysteine mutants, half-life

* selected for oral presentation

† not represented at ISFP Congress
Expression and purification of cysteine mutants of PAI-1 with very long half-lives

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The mutation of amino acids, Gln197, Gly355?Cys, produce a PAI–1 with a very long half–life of over 700 h as reported previously. However, VLHL PAI–1 was expressed in E. Coli with a modest yield of 1 mg–purified protein in 1L of cell culture. To increase the yield, we expressed VLHL PAI–1 in baculovirus. The cDNA encoding of PAI–1 was excised from the VLHL PAI–1 plasmid as a Ndel/Xho fragment. Afterwards, the PCR product of the VLHL PAI–1 Ndel/Xho I fragment was ligated into the pFastbac plasmid containing a 6His purification tag. VLHLNS PAI–1 mutation (at P1, Arg369?Ala) was introduced by PCR and the gene was transferred into the baculovirus vector in the same way as VLHL PAI–1. We assumed that the VLHLNS construct would remain in an active conformation as VLHL PAI–1 does, but would not have any inhibitory activity toward uPA. We used the baculovirus expression system, which promises ~20 mg of protein from 1L of cell culture. In the pFastbac vector, the expression of the gene is controlled by the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV), polyhedrin (PH), or the p10 promoter for high–level expression in insect cells. The plasmids were transposed into a recombinant bacmid with the help of DH10Bac E. coli cells (Invitrogen), which contain a baculovirus shuttle vector (Bacmid) with a min–attTn7 target site and a helper plasmid. The recombinant bacmid DNA was isolated from the white colonies grown for 48 h at 37°C on a LB agar plate containing 50mg/L kanamycin, 7mg/L gentamycin, 10mg/L tetracycline, 100mg/L X–gal and 40mg/L, IPTG and which was used to transfect SF9 cells derived from Spodoptera Frugiperda (Fall Armyworm) by cellfectin reagent (Invitrogen). The virus was amplified to ~ 2x107 plaque forming units (pfu)/mL and was added to SF9 cells (~2x106/mL) in 6 well culture plates. The plates were incubated at 27°C for different time intervals. The virus was subsequently amplified to ~ 2x108 plaque forming units (pfu)/mL and was used to infect SF9 cells (~2x106/mL) on a large scale (1L cell culture). The cells were harvested and lysed by two freeze–thaw cycles. The lysate was then centrifuged at 3,000xg for 20 minutes to pellet the cellular debris. The supernatant was transferred to a fresh tube for purification. The supernatant was loaded onto a column packed with nickel resin (Invitrogen) at a flow rate of 0.4 ml/min and the column was then washed with a buffer containing 40 mM imidazole in 20mM Hepes buffer pH 8.0, and protease inhibitors until no washed proteins were detected. The protein was then eluted using a gradient of 40 – 250 mM imidazole in native buffer. The peak fractions were dialyzed to remove imidazole and concentrated to a desired concentration. VLHL PAI–1 was active against uPA, however VLHLNS PAI–1, as expected, was not. Most likely, both PAI–1s are in an active conformation. Purity was determined as +95%.

Keywords: PAI–1, mutation, cysteine
ROLE OF PLASMINOGEN ACTIVATOR INHIBITOR-1 AND UROKINASE IN EARLY STAGES OF ATHEROGENESIS IN KNOCKOUT MICE

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Plasminogen activator inhibitor–1 (PAI–1) and urokinase (uPA) and have been detected in human atherosclerotic plaque and may contribute to the pathogenesis of atherosclerosis by supporting macrophage recruitment and migration into the vessel wall. Previous studies in genetically modified mice have focused on the characterization of the late stages of plaque formation, when the plaque size does not depend solely on cell migration, but rather on extracellular matrix deposition. The purpose of this study was to examine the impact of uPA and PAI–1 on macrophage adhesion and penetration in the lesion–prone areas (intercostal artery branches) of apolipoprotein E (apoE–/-) deficient mice, during the early stages of diet–induced atherogenesis. For this purpose we setup a novel approach to image macrophage recruitment and migration into the mouse arterial wall. Mice with single (apoE–/-) or combined apoE and uPA (apoE–/- : uPA–/-) or PAI–1 (apoE–/- : PAI–1–/-) genetic deficiencies were fed an atherogenic diet for 1 to 4 weeks. Aortic segments were permeabilized and stained for the macrophage marker Mac3 antigen by immunofluorescence on whole–mount preparations, followed by deep tissue confocal or multiphoton microscopy and 3D image rendering. This approach allowed us to detect the incipient lesions formed at branch ostia, as early as one week post–atherogenic diet. Taking advantage of the autofluorescence of the elastin, it was possible to image macrophage–dependent matrix degradation at single–cell level. Our results demonstrate that apoE–/- : PAI–1–/- mice have a significantly increased macrophage accumulation at the branch ostia, comparing to apoE–/- mice, both in animals fed an atherogenic diet (16 ±1.4 vs. 6.5± 1.2 cells/lesion; p<0.01), and in those fed a standard chow (9±0.8 vs 1.2 ±0.73 cells/lesion; p<0.01) at 2 weeks. In contrast, apoE–/- : uPA–/- did not show significant differences in the number of macrophages accumulated at branches, comparing to controls (7.5±1.05 vs. 6.5 ±1.1 cells/lesion, p>0.05, 2 weeks, atherogenic diet; 2.22±0.7 vs. 1.2±0.73 cells/lesion, p>0.05, 2 weeks, standard chow), but the cells were mainly adherent to the vascular surface, rather than penetrated into the vessel wall. These data demonstrate that PAI–1 deficiency increases macrophage accumulation in the incipient atherosclerotic plaque and macrophage dependent matrix degradation. The lack of urokinase did not significantly affect the macrophage adhesion at branches but clearly inhibited leukocyte penetration into the vessel wall. These data demonstrate a regulatory role of PAI–1 on inflammatory cell migration by inhibiting plasmin–dependent proteolysis.

Keywords: atherogenesis, PAI–1, urokinase

* selected for oral presentation
Vitronectin accelerates protease inhibition by PAI-1 through its cryptic protease binding site

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Vitronectin binds and stabilises PAI-1’s activity through its somatomedin B (SMB) domain and accelerates PAI-1’s protease (thrombin and activated protein C) inhibition by more than 100-fold. To investigate the mechanism underlying this acceleration, we prepared the SMB domain alone as well as various SMB containing fragments of vitronectin. Kinetics studies showed that these fragments of vitronectin, like urea-treated vitronectin, did not promote PAI-1’s protease inhibition. Cross-linking studies with an inactive thrombin variant (S195A) showed that thrombin could only be cross-linked to native VN in the presence of active PAI-1. Mutagenesis studies of thrombin showed that substitution of the surface exposed residue Asp100 with Arg attenuated vitronectin–PAI-1’s inhibition of thrombin by more than 50%, while other mutations of thrombin such as Glu97Arg and Asp178Arg etc had little effect. Altogether, these data indicate that native vitronectin has a cryptic thrombin binding site, which is exposed upon PAI-1 binding (likely to be masked in urea treated VN) and interacts with the thrombin surface near Asp100, and that vitronectin accelerates PAI-1’s protease inhibition by bridging PAI-1 and thrombin together.

Keywords: PAI-1, Vitronectin, Thrombin
PAI-1 modulates the inflammatory response during Lipopolysaccharide (LPS)-induced endotoxemia.

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Background: Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of plasminogen activation. Plasma PAI-1 level rises during sepsis and exerts a worse prognosis. PAI-1 participation to sepsis has been poorly documented and was mainly associated with fibrin deposits. Beside fibrin accumulation, increased tissue PAI-1 expression may participate to the poor outcome of endotoxemia through other mechanisms. Interestingly, cell associated plasminogen activation may be involved in extravasation and migration of activated T cells. Moreover, PAI-1 is able to control the generation of active TGFbeta1 which participate to differentiation of regulatory T cells (Treg) capable of suppressing inflammatory response.

Methods and results: We examined the role of PAI-1 in transgenic mice that either lack or overexpress the murine PAI-1 gene (PAI– or PAI+) on systemic inflammation and behavior of lung leukocytes populations during LPS challenge. In the basal condition, when compared to wild-type mice (WT) PAI+ exhibited 11 and 15 fold higher plasma and lung PAI-1 levels, respectively. No such difference was observed in the liver or the kidney. Survival of PAI-1+ following LPS challenge was reduced compared to WT and was not affected in PAI-1–. The survival rate prompted us to examine the inflammatory state 4 hours after LPS injection. Compared to WT, PAI-1+ mice exhibited significantly higher plasma levels of TNF, IL6 and MIP-1alpha. Leukocytes populations were examined in lungs. CD45+ (leukocytes) and Ly6G+ (polynuclear neutrophils) cells infiltration was not different between the groups. F4/80 macrophages infiltration was significantly reduced in PAI-1+. CD25+ cells infiltration was totally blunted in PAI-1+ (ratio LPS/placebo, WT vs PAI+: 3.4 vs 1 p<0.0001). In parallel, Foxp3 mRNA, a specific marker of Treg, was 1.8 fold lower in PAI-1+ than in WT lungs (p<0.05). Active TGFbeta1 required to maintain Treg suppressive function, was lower in PAI-1+ (15±1.4 vs 21±1.7 ng/mg protein). In addition, PAI-1+ lungs showed larger fibrin deposits (0.15±0.02 vs 0.03±0.01% of the total area; p< 0.01) and greater extent of fibrosis (22.1±1.5 vs 16.5±1.5% of the total area; p<0.05) than WT. None of these results were observed after placebo injection. Opposite results were obtained for most of the studied parameters in PAI– mice.

Conclusion: These results suggest that PAI-1 is an important determinant of the inflammatory response during sepsis partly through the control of Treg recruitment or differentiation.

Keywords: PAI-1, Inflammation

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Defining the Second Site for Interaction of PAI-1 and Vitronectin

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Vitronectin is a versatile protein capable of binding a number of ligands, endowing it with different properties depending on its location in either the circulation or extracellular matrix. In both of these contexts, one of the most important ligands for vitronectin is the serine protease inhibitor plasminogen activator inhibitor-1 (PAI-1). This interaction stabilizes active PAI-1 while inducing conformational changes in vitronectin, leading to a more adhesive form of vitronectin. A well characterized high-affinity binding site for PAI-1 is located within the amino-terminal somatomedin B domain of vitronectin. Several recent studies have revealed a second, somewhat weaker PAI-1 binding site within vitronectin. Monoclonal antibodies have tentatively mapped this second site to an acidic, flexible linker region that connects the somatomedin B domain to the central beta-propeller domain of vitronectin. To directly evaluate binding to this putative second site, a deletion mutant of vitronectin with the somatomedin B domain removed (delta sBVN) was engineered. A comprehensive survey of vitronectin functions determined that the short deletion had no effect on heparin-binding, integrin-binding or cellular adhesion. Binding to the urokinase receptor was completely abolished, whereas delta sBVN retained the ability to bind to wild-type PAI-1. Furthermore, a stoichiometric complex of PAI-1 and the purified somatomedin B domain retained binding to the residual site in delta sBVN. This observation demonstrates a vitronectin-binding site in PAI-1 that complements the second PAI-1-binding site in vitronectin. Using surface plasmon resonance, a panel of PAI-1 mutants was screened for their ability to bind delta sBVN. This data was supported by the ability of PAI-1-specific antibodies to disrupt binding to delta sBVN. Based on the binding experiments, the secondary vitronectin-binding site was localized on PAI-1. Compared to wild-type PAI-1, the mutant R115-118E exhibits a 10-fold reduced binding to delta sBVN. Mutant forms of PAI-1 with other amino acid substitutions proximal to these residues also exhibited reduced binding to delta sBVN, suggesting the region near Helix D is important for mediating binding to the second site on vitronectin. Other residues that comprise this secondary vitronectin-specific site in PAI-1 include R76, K80, W86, R115 and R118, defining a substantial positively charged character for the binding surface. Consistently, analytical ultracentrifugation on the PAI-1:vitronectin complex demonstrated that increasing NaCl concentration favors 1:1 versus 2:1 PAI-1:vitronectin complexes and hampers formation of higher-order complexes. These results support a model for ordered assembly of PAI-1:vitronectin complexes via two distinct binding sites, leading to formation of higher-order multimers that may regulate the tissue-distribution of vitronectin.

Keywords: vitronectin, PAI-1, uPAR
High PAI-1 and the malignant phenotype: exogenous PAI-1 signals to tumors cells via uPA/uPAR/LDLR-family system and ERK1/2 pathway inducing increased adhesion and integrin redistribution

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In a clinical setting high PAI-1 levels of a tumor frequently correlate with the grade of malignancy of the disease. The basis for this phenomenon is not easily explained and might differ for different tumors. In an animal model using nu/nu mice, we could show previously that HT-1080 subclones selected in vitro according to their PAI-1 secretion exhibited in vivo in a lung colonization assay a pattern where PAI-1 secretion correlated with the number of lung “metastases”. Moreover, when a low PAI-1 secreting HT-1080 clone was stable transfected with PAI-1, again a correlation with the number of lung colonies was found; consistently, also in vitro adhesion on several artificial matrices positively correlates with PAI-1 expression. The increase in adhesion of PAI-1 transfected HT-1080 clones was RGD-dependent, indicating its integrin dependency. To analyze the mechanism for PAI-1 induced integrin dependent adhesion, we found that sustained high PAI-1 levels influenced integrin expression and redistribution within the cell promoting a shift from a static/stationary towards a migratory/invasive phenotype. Analysis of integrin mRNA with QT-PCR and of protein by FACS revealed an overall quantitative decrease of different integrin subunits accompanied by redistribution of the alpha-v partner beta-integrin subunits to the cell surface in the PAI-1 overexpressing cells. This increase in adhesion was only seen after long-term exposure of low PAI-1 expressing cells to exogenous PAI-1 or in cells overexpressing PAI-1. With respect to the mechanism by which PAI-1 can affect integrins in these cells we found using reporter assays significant activation of the MEK/ERK pathway by PAI-1. This activation was not transient but showed a sustained pattern with p44/42 phosphorylation still seen after 60 minutes and the presence of active ERK1/2 in the nucleus in cells exposed to PAI-1. PAI-1 can induce signaling through the respective surface receptor of PA–PAI–1 complex (uPAR, LDLR-family members) involving eventually further surface molecules (e.g., integrins, M6PR). Reporter gene assays and western blotting experiments revealed that sustained activation of the MEK/ERK pathway by PAI-1 required both uPAR and internalization by a LDLR-family member, since p44/42 phosphorylation were prevented by the high affinity LDLR-family ligand RAP and was absent in cells deficient in uPA or uPAR and was inhibited when endocytosis was prevented. To prove that PAI-1 induced MEK/ERK activation is also responsible for PAI-1 induced adhesion changes, we determined that PAI-1 dependent enhanced in vitro cell adhesion was reverted in the presence of RAP and was mimicked by cells overexpressing ERK1. These cells showed increased matrix adhesion and a distribution pattern of integrins resembling that in cells overexpressing PAI–1 or being exposed to exogenous PAI–1. In conclusion, PAI–1 along with uPA, uPAR, and a LDLR-family member is capable of triggering intracellular signaling inducing a sustained type of ERK1/2 activation, which in turn alters integrin function influencing adhesive properties and thereby modulating the invasive and metastatic phenotype of these cells. This would explain why malignant cells exposed to a high PAI–1 environment would exhibit a more “malignant” phenotype.

Keywords: PAI–1, signaling, cancer
PLAP/IRAP deficiency attenuates hypofibrinolysis in mice thrombosis models

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Reduced fibrinolytic activity is associated with adverse cardiovascular events. Although Insulin-Regulated Aminopeptidase (IRAP) was recently identified as angiotensin (Ang) IV receptor (AT4R), it remains unknown how much this Ang IV-AT4R signaling distal to Ang II affects PAI-1 activation in the fibrinolytic process. To test hypothesis that AngIV would inhibit fibrinolysis via PAI-1 activation, we evaluated the degree of fibrinolysis in thrombosis models using IRAP knockout mice (AT4R−/−).

Methods and Results: AT4R−/− mice showed normal glucose levels (68±6 mg/dl, mean±SD) and no difference was seen in visceral fat weight if compared with wild type C3H mice (WT). We tested the level of PAI-1 mRNA expression in mouse endothelial cells (ECs) from WT and AT4R−/− (n=3 each). In ECs from WT, AngIV treatment (10−9 to 10−6 M) as well as AngII treatment (10−9 to 10−6 M) increased PAI-1 mRNA expression in a dose dependent manner, while AngII, but not AngIV, increased PAI-1 mRNA in ECs from AT4R−/−. To assess fibrinolysis in an acute thrombosis model, renal glomerular fibrin deposition was counted after lipopolysaccharide injection (0.2mg/kg, i.v., n=5). Fibrin deposition was reduced in AT4R−/− as compared to WT at 4 hrs (12±3% of total gromeluli vs. 27±4%, p=0.012). The levels of plasma active PAI-1 antigen measured by ELISA were also reduced in AT4R−/− than those of WT (120±31 vs. 62±12 ng/mL, p<0.001). Western blotting analysis demonstrated that NF-κB expression in the livers was suppressed in AT4R−/− by 43% as compared to WT. For a chronic thrombosis model, thrombus formation was observed over 7 days after carotid artery ligation and cuff placement. In WT, 100% of thrombus occlusion was observed, while 67% in AT4R−/− (p<0.01, n=12 each) in accordance with suppressed mononuclear cell infiltration. Conclusions: In acute and chronic thrombosis models, disruption of AngIV–AT4R signaling leads to accelerated fibrinolysis with decreased PAI-1 expression, suggesting that AT4R is a novel therapeutic target against hypofibrinolysis in thrombotic diseases.

Keywords: PLAP/IRAP, Angiotensin IV, PAI-1
**PAR’s**

**ID: 95**

**APC and duodenase can control an inflammation via the PARs on mast cells**

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Proteinases play key roles in homeostasis, inflammation, cardiovascular and gastric disease. They activate cells, involved in these processes, via the proteinase-activated receptors (PARs). Four members of the PARs family have been recognized: PAR1, 3, 4 are targets for thrombin; PAR2 is a receptor for trypsin, mast cell tryptase and other high-affinity agonists. Activated protein C (APC, an anticoagulant serine proteinase) and duodenase (a serine proteinase of duodenum) are probable low-affinity agonists for PAR1 (PAR1-AP) and duodenase is a possible PAR1-antagonist, as cathepsin G. Certain proteinases, including thrombin, APC, duodenase, are involved in inflammation and can contact with mast cell at sites of tissue and vessel damage, because they localized along blood and lymphatic vessels. Data about role of thrombin in inflammation are contradictory. Moreover data about the APC and duodenase effects on mast cell are absent. The effects of APC, duodenase, thrombin and PAR1-AP on peritoneal mast cell secretion were investigated to reveal PAR1-mediated events in inflammation. In a model of acute inflammation in rats (intraperitoneal injection of thioglycolate broth) occurrence of both, thrombin and APC, in the peritoneal cavity was shown. Kinetic analyses revealed the maximum release of thrombin on 30 min and APC on 120 min of inflammatory process. Mast cells, involved in inflammation, release a wide range of proinflammatory, vasoactive mediators, beta-hexosaminidase, proteases (tryptase), cytokines and other factors in response to activation. In vitro experiments have shown that duodenase (0.1–80nM) as well as thrombin (1–100nM) activates peritoneal mast cell in dose-dependent manner and accelerates beta-hexosaminidase release. Maximum release of mediator (36.7±1.35%) was reached at 80nM duodenase. Duodenase action on mast cell was inhibited by 59.7% by soybean trypsin inhibitor. Interestingly, mast cell pretreatment with 8nM duodenase reduced thrombin (10nM) and PAR1–AP (400mkM) stimulated cell degranulation on 35.1 and 41.7%, respectively. Thus, duodenase like to cathepsin G could be an antagonist of PAR1. Also we have shown that duodenase can reduce PAR1–AP–provoked platelet aggregation, that confirm our hypothesis about PAR1–mediated effect of duodenase. In contrast with duodenase action, APC at a very low concentrations (0.5, 1 and 2.35nM) was able to reduce the rest mast cell mediator release (5.9, 9.3 and 11.0%, P<0.01) concerning with spontaneous level (13.0%). Not only rest mast cell but also cells, activated with compound 48/80 (well known mast cell degranulator) responses to APC (0.5–2.3nM) with reduction of beta–hexosaminidase release. The L-NAME pretreatment of mast cell abolished this APC effect. Thus, APC–induced diminish of mediator release could be accounted for NO generation in mast cell. Decrease of mast cell degranulation by APC could be evidence of its anti-inflammatory properties. In conclusion, for the first time our study has shown that APC regulates mast cell activation and this effect may be mediated via PARs. Serine proteinase duodenase may act both as activator and as terminator of PAR1, imitating cathepsin G effect. Probably APC and duodenase take part in regulation of inflammation in vivo.

**Keywords:** PARs, inflammation, activated protein C
Regulation of cancer cell plasmin generation by annexin A2-S100A10 heterotetramer (AIIt)

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Many findings over several decades strongly suggest an important role for the plasminogen activation system in cancer cell invasion and metastasis. We have investigated the role of S100A10, annexin A2 and the heterotetrameric complex formed by these subunits (AIIt) in plasminogen regulation. Surface plasmon resonance studies established that AIIt immobilized on a phospholipid coated biosensor chip, bound tPA, plasminogen and plasmin. Immobilized S100A10 bound directly to tPA, plasminogen and plasmin whereas immobilized annexin A2 bound only plasmin. In another series of in vitro experiments we observed that the formation of the S100A10–plasmin complex but not the annexin A2–plasmin complex protected plasmin from inactivation by alpha2-antiplasmin. We also observed that incubation of plasminogen with tPA and AIIt or S100A10 dramatically stimulated the plasminogen activator–dependent conversion of plasminogen to plasmin in vitro. In contrast, annexin A2 minimally stimulated plasminogen activation. Site-directed mutagenesis studies have shown that the carboxyl-terminal lysines of S100A10 are critical for plasminogen binding and tPA–dependent plasminogen activation. We have also utilized both antisense and siRNA methodologies to knockdown extracellular S100A10 levels. We demonstrated that the knockdown of S100A10 did not affect the annexin A2 levels. Loss of extracellular S100A10 resulted in a 70–90% loss in cellular plasmin generation despite the fact that annexin A2 levels were unchanged. The S100A10 knockdown cells were also less invasive and showed a dramatic loss in tumor formation and metastatic potential. These studies provide direct evidence for the importance of S100A10 in cellular plasmin generation. We have also examined the question of whether AIIt is proteolyzed during stimulation of plasmin generation. We observed that the addition of plasminogen to cancer cells did not cause the proteolysis of AIIt but resulted in the oxidation of both annexin A2 and S100A10. End product analysis established that AIIt stimulated autoproteolysis of the plasmin Lys468–Gly469 bond and also catalyzed the reduction of the plasmin Cys462Cys541 disulfide. These two reactions resulted in the release of angiostatin (Lys78–Lys468) from plasmin and the oxidation of AIIt. The plasminogen–dependent oxidation of AIIt could be attenuated by thioredoxin. We also showed that thioredoxin reductase catalyzed the transfer of electrons from NADPH to the oxidized thioredoxin, thus completing the flow of electrons from NADPH to AIIt. Thus, AIIt acts as a plasmin reductase catalyzing the release of angiostatin from plasmin. Collectively, our results define the role of S100A10 and annexin A2 in plasminogen regulation. S100A10 is a key regulator of cell surface plasmin generation whereas the S100A10–annexin A2 complex regulates plasmin autoproteolysis and angiostatin generation. Funded by the Alberta Cancer Board, CIHR, and Alberta Heart and Stroke Foundation.

Keywords: annexin A2, S100A10, plasminogen
Plasminogen receptors on macrophages: role of extracellular histone 2B as a plasminogen receptor

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Localization of plasminogen/plasmin to the cell surface is critical in facilitation of plasminogen (Plg) mediated proteolytic degradation during leukocyte migration to sites of inflammation. Plg preferentially binds to cell surface proteins, which have exposed C-terminal lysine. However, the critical Plg receptors (PlgRs) that are involved in Plg binding to cells and hence Plg-mediated cell migration during inflammation remain uncertain. We have investigated the expression of three previously characterized PlgRs, alpha-enolase, annexin II and p11, on the surface of two mouse macrophage cell lines (RAW264.7 and J774A.1) and on thioglycholate induced mouse peritoneal macrophages. In addition, we have also characterized the cell surface localization and contribution of histone H2B, a newly identified PlgR, in Plg-mediated cell functions. The cell surface localization of H2B, which is primarily a nuclear protein, of mouse peritoneal macrophages and macrophage cell lines was confirmed by cell surface biotin labeling and confocal microscopy. The interaction of Plg with H2B was C-terminal lysine dependent as confirmed by carboxypeptidase B sensitivity. Plg binding to J774A.1 cells was substantially higher than to RAW264.7 cells, and these cells express higher levels of cell surface H2B, alpha-enolase, annexin II and p11. Moreover, total Plg binding and H2B, alpha-enolase, annexin II and p11 expression levels were upregulated on monocytes recruited in response to thioglycholate versus peripheral blood monocytes of mice. Using Fab fragments of specific anti-alpha-enolase, anti-H2B, anti-annexin II and anti-p11, we found that H2B contributes ~ 45–50% of total in-vitro Plg binding and whereas alpha-enolase, annexin II and p11 has only 20–25% contribution to total Plg binding to mouse macrophages. Furthermore, intravenous injection of anti-H2B Fab reduced total monocyte recruitment into peritoneum of thioglycholate treated mice by 44% compared to nonimmune Fab injected mice. Fab against another PlgR, alpha-enolase, impaired recruitment of monocytes by only 22%. Taken together, these data suggest that multiple PlgR contribute to Plg binding to macrophages, and among these, H2B plays a very prominent role.

Keywords: Plasminogen receptor, Histone 2B, macrophage
Requirement of Matrix Metalloproteinase-9 Activation in Plasminogen-regulated Macrophage Recruitment

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Requirement of Matrix Metalloproteinase-9 Activation in Plasminogen-regulated Macrophage Recruitment

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Dept. Molecular Cardiology, Cleveland Clinic Lerner Research Institute, 9500 Euclid Ave., Cleveland, OH. The importance of inflammation in the initiation and development of cardiovascular diseases (CVD) has been well established, and understanding the molecular basis of inflammation in CVD will lead to new therapeutic strategies. The plasminogen (Plg) network, a primary enzymatic pathway regulating fibrinolysis in thrombosis and hemostasis, is a major contributor to the regulation of leukocyte migration in inflammation. Our previous data showed that Plg deficiency (Plg−/−) significantly decreases (65%) macrophage recruitment in a widely used thioglycollate-induced peritonitis mouse model, but the underlying mechanisms remain unclear. Our results suggest that Plg deficiency impairs macrophage migration across tissue, as indicated by the large number of macrophages that accumulate in peritoneum in Plg−/− mice 72h after thioglycollate stimulation (peak of macrophage migration). Extensive accumulation of the macrophages in the extra-cellular matrix (ECM) of the peritoneum was observed in Plg−/− mice (45.7 ± 2.7%, n=4, % of total area), compared with to WT mice (11.6 ± 1.2%, n=4). Moreover, 72h after thioglycollate stimulation, in Plg−/− mice, collagen deposition was significantly (P <0.01) increased (4.7% ± 0.6, n=4) in the peritoneum compared with WT mice (1.0 ± 0.2%, n=4) which suggested ECM degradation is involved for Plg-regulated macrophage migration. Furthermore, our data demonstrate that matrix metalloproteinase–9 activation is required for the Plg-mediated macrophage migration in vivo and in vitro. First, MMP–9 was activated in peritoneal lavage of WT but not Plg−/− mice 72h after thioglycollate stimulation by gelatin zymography. Second, administration of MMP–9 (50 mg/kg ip.) efficiently restored macrophage recruitment in Plg−/− mice (increase by 57%). Third, thioglycollate–induced macrophage recruitment was significantly (P < 0.01) inhibited by 59% with MMP–9 antibody neutralization (4 mg/kg, ip.) in WT mice. In MMP–9 deficient mice, thioglycollate–induced macrophage recruitment was significantly (P < 0.05) inhibited by 67%. In addition, an in vitro macrophage migration assay showed that MMP–9 neutralization abolished (by 93%) the Plg–induced macrophage migration across Matrigel, a complex ECM. In conclusion, the data presented here verify a major role of Plg in inflammatory cell recruitment and suggest that this role is dependent on its regulation of MMP–9 activation and ECM degradation.

Keywords: Plasminogen, Matrix Metalloproteinase–9, Macrophage migration
An antibody anti-alpha-enolase, MAb 11G1, inhibits myogenesis in vitro and in vivo

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The plasminogen activation (PA) system is an extensively used mechanism for the generation of proteolytic activity in the extracellular matrix (ECM), where it contributes to tissue remodeling in a wide range of physiopathological processes. Plasmin is an extracellular protease specialized in the degradation of extracellular matrix components. Plasmin is generated by activation of plasminogen by urokinase–type plasminogen activator (uPA) and by tissue–type plasminogen activator (tPA). Alpha–enolase constitutes a receptor for plasminogen in several cell lines, serving to focalize proteolytic activity on the cell surface. Increasing evidence is accumulating about the important role of the PA system in the homeostasis of muscle fibers and their surrounding ECM. We, among others, have shown a role for uPA, plasminogen and type–1 plasminogen activator inhibitor in myogenesis in vitro and in vivo. Preliminary results of our laboratory have shown that alpha–enolase was up–regulated in a myogenic cell line upon differentiation, but the role of alpha–enolase as a plasminogen receptor deserves further analysis. We have used inhibitors of plasminogen/alpha–enolase binding to evaluate the importance of this interaction in myogenesis: MAb 11G1, a monoclonal antibody against alpha–enolase, that inhibits plasminogen binding to alpha–enolase and its further activation to plasmin; and e–aminocaproic acid (EACA), a lysine analog that also inhibits plasminogen binding to alpha–enolase. We have also used aprotinin, an inhibitor of plasmin activity. Measuring the expression of myogenic markers as myogenin and developmental myosin heavy chain (dMHC), our results indicated that MAb 11G1 abrogated differentiation and fusion of primary cultures of muscle precursor cells (MPCs). This study was developed by RT–PCR, Western–blot and immunocytochemistry. Additionally, we found that migration ability of MPCs was also dramatically reduced by MAb 11G1, using Boyden Chamber assays. EACA showed the same inhibitory effect as MAb 11G1, while in contrast, aprotinin had no effect in MPCs differentiation, fusion or migration. To study the importance of plasminogen/alpha–enolase binding in skeletal muscle regeneration in vivo, we used two animal models: an injury–induced model, by injection of cardiotoxin (a natural myotoxin), and mdx dystrophic mice (the animal model for Duchenne Muscular Dystrophy). Inhibitors (MAb 11G1, EACA and aprotinin) were injected daily intraperitoneally and muscle regeneration parameters were evaluated. The results obtained showed that the regeneration (measuring regenerating areas and necrotic tissue) in injury–induced model was abrogated in animals treated with MAb 11G1 and EACA. Also mdx mice presented a more severe dystrophinopathy when treated with MAb 11G1 or EACA. In contrast, aprotinin treatment had no effect in both animal models. Since only inhibitors of plasminogen/alpha–enolase binding have an effect on MPCs differentiation and muscle regeneration, our results demonstrate that plasmin activity is necessary for myogenesis to take place correctly, in an alpha–enolase dependent way. Plasminogen/alpha–enolase binding therefore could be an important target in the development of treatments for Duchenne Muscular Dystrophy.

**Keywords:** alpha–enolase, plasminogen receptor, myogenesis
The Effects of a PAM-derived Peptide on the Structure and Zymogenicity of Plasminogen

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The infectivity, virulence, and invasiveness of group A streptococci (GAS) is largely mediated by the ability of these pathogens to bind host plasminogen (Pg) at the cell surface where it is activated by GAS-secreted streptokinase (SK). The resulting bacteria-bound plasmin (Pm) can inhibit fibrin encapsulation and degrade extracellular matrix components, thus facilitating systemic infection. The GAS-surface protein responsible for Pg recruitment is the Pg-binding group A streptococcal M protein (PAM), which utilizes the lysine binding site (LBS) of Pg kringle 2 for docking. The kringle 2–binding segment of PAM has been localized to two tandem repeats in its N-terminal region. A 30 residue helical peptide (VEK–30) overlapping this repeat region can recapitulate virtually all of the high-affinity binding associated with the full length molecule and is an excellent vehicle for probing the molecular basis of Pg–PAM interactions. Our recent crystal structure of Pg kringles 1–3 complexed with VEK–30 reveals that the latter employs a pseudo–lysine moiety–wherein Arg and Glu side chains are separated by one helical turn–to bind within the bipolar LBS of kringle 2. This structure also demonstrates that significant rearrangement of the kringles occurs upon VEK–30 binding. This suggests a model for Pg activation, in vivo, wherein Pg is rendered more vulnerable to SK activation by virtue of PAM–induced conformational reorientation. To address this possibility, we have examined the effect of VEK–30 on the activation of Pg by both uPA and SK. We have found that VEK–30 accelerates Pg activation in a concentration–dependent manner. These results, coupled with sedimentation velocity data acquired in the presence and absence of VEK–30, contribute to a clearer understanding of the molecular events attending the dissemination of GAS infection.

Keywords: plasminogen, VEK–30, Group A streptococci
The annexin A2 heterotetramer is a receptor for the plasmin-induced signaling in human peripheral monocytes activated by proteolytic cleavage

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We have previously demonstrated that plasmin acts as potent proinflammatory activator of human peripheral monocytes leading to activation of protein kinase cascades and a number of transcription factors. An uncompromised proteolytic activity was essential for the plasmin–mediated monocyte activation pointing to the necessity of a proteolytic activation of the signaling receptor. Despite considerable efforts, little progress has been made so far in the identification of a signal–transducing plasmin receptor. Here we identify the annexin A2 heterotetramer, composed of annexin A2 and S100A10, as receptor for the plasmin–induced signaling in human monocytes. Monocytes express the annexin A2 heterotetramer on the cell surface as shown by i) flow cytometry, ii) fluorescence microscopy, and iii) co-immunoprecipitation of biotinylated cell surface proteins. Binding of plasmin to annexin A2 and S100A10 on monocytes was verified by biotin transfer from plasmin labeled with a trifunctional cross-linker. Antibodies directed against annexin A2 or S100A10 inhibited the chemotaxis elicited by plasmin, but not that induced by the standard chemoattractant fMLP. A control antibody directed against the thrombin receptor PAR–1 had no effect on the plasmin–mediated chemotaxis suggesting that PAR–1 is not involved. Further, downregulation of the annexin A2 or S100A10 expression in monocytes by antisense oligodeoxynucleotides impaired the chemotactic response to plasmin, but not that to fMLP. Antisense oligodeoxynucleotides similarly decreased the TNF-alpha release by plasmin– but not by LPS–stimulated monocytes. At the molecular level, stimulation with plasmin, but not with catalytically inactivated plasmin, induced cleavage of annexin A2 and dissociation of the annexin A2 heterotetramer complex. In contrast to annexin A2, S100A10 is not a substrate for plasmin. Substitution of lysine to alanine in position 27 abolished the cleavage of recombinant annexin A2 in vitro indicating a single cleavage site. These data shed new light on the plasmin–induced signaling; taken together they identify the annexin A2 heterotetramer in human monocytes as a signaling receptor activated by plasmin via proteolysis.

Keywords: Plasmin, Signaling, Monocytes
Molecular requirements for modulation of NMDA receptor signaling by tissue-type plasminogen activator

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Background and Objective: We have previously shown that tissue type plasminogen activator (t-PA) potentiates NMDA receptor (NMDAR) signaling by proteolysis at arginine 260 of the amino terminal domain (ATD) of the NR1 subunit (NR1). The molecular background of this action has been elucidated using recombinant rt-PA and the structurally-related plasminogen activators, desmoteplase, reteplase, and tenecteplase.

Methods: Cleavage of ATD-NR1 was examined using rATD-NR1, a His-tagged recombinant form, as an enzyme substrate, immunoblot analysis being performed post-incubation of rATD-NR1 with a plasminogen activator. Additionally, the binding of plasminogen activators to rATD-NR1 was analyzed using surface plasmon resonance (SPR).

Results: Cleavage of the rATD-NR1 was observed with rt-PA, reteplase and tenecteplase, but not desmoteplase, which was the only compound tested that possesses no kringle2 domain. Rt-PA bound to rATD-NR1 in a concentration-dependent and reversible manner consistent with a two-site kinetic model (KD = 24 nM). Analysis indicated that two binding steps are required for cleavage of the NR1 subunit, i.e. kringle2 binding as the first step and binding of the catalytic domain as the second step. Tenecteplase bound to rATD-NR1 with characteristics similar to those of rt-PA and with a reliable fit to two-site kinetics. Binding of reteplase occurred with a pattern similar to that of tPA and TNK, but with a lower apparent affinity. By contrast, no binding was obtained when desmoteplase was tested.

Summary: We have found that rt-PA binds ATD-NR1 by a two-site system involving sequential binding of its kringle2 and catalytic domains. Only plasminogen activators carrying a kringle2 domain were able to cleave the rATD-NR1. Thus, this domain appears to be critical for t-PA-mediated enhancement of NMDAR signaling. Our data suggest that strategies able to prevent or avoid kringle2-dependent enhancement of NMDAR signaling may allow for thrombolysis without neurotoxicity.

Keywords: tissue plasminogen activator, NMDA receptor, desmoteplase

* selected for oral presentation  
† not represented at ISFP Congress
Substrate profiling of the Clan CE proteases

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Human Clan CE contains 7 family members, also known as SENPs (Sentrin/SUMO–specific proteases). These enzymes play a similar role to deubiquitinylating enzymes by participating in the control of Ub–like modifiers. In humans, Ub–like modifiers set a group of at least 12 members among which the best characterized is the SUMO family. Recent studies show that SUMO–1, SUMO–2 and SUMO–3 are putative natural substrates of most SENPs. SENP8 has less similarity with other family members and demonstrate activity only against NEDD8 modified proteins. However, still very little is known about the substrate specificity of SENPs. To investigate the minimal substrate requirement and specificity of SENPs, we have synthesized a group of synthetic substrates in the form of 1 up to 5 amino acid residues of peptide fluorogenic (AFC) molecules. The structures of substrates were based on sequences of all known SUMOs – SUMO–1, SUMO–2, SUMO–3 and SUMO–4.

Keywords: SENP, SUMO, cysteine protease
Urokinase signalling through its receptor protects against anoikis by increasing Bcl-xL expression levels

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The acquired capabilities of resistance to apoptotic cell death and tissue invasion are considered to be obligate steps in tumor progression. The binding of the serine protease urokinase (uPA) to its receptor (uPAR) plays a central role in the molecular events coordinating tumor cell adhesion, migration and invasion. We investigated whether uPAR signalling may also prevent apoptosis following loss of anchorage (anoikis) or DNA damage. If non-transformed human retinal pigment epithelial cells (RPE) are pre-exposed to uPA or to its non-catalytic amino-terminal region (residues 1–135), they exhibit a markedly reduced susceptibility to anoikis as well as to UV–induced apoptosis. This anti-apoptotic effect is retained by an uPA–derived synthetic peptide corresponding to the receptor binding domain and is inhibited by anti–uPAR polyclonal antibodies. Furthermore, the stable reduction of uPA or uPAR expression by RNA–interference leads to an increased susceptibility to UV–, cisplatin– and detachment–induced apoptosis. In particular, the level of uPAR expression positively correlates with cell resistance to anoikis. The protective ability of uPA is prevented by UO126, LY294002, by a MAPK–targeting siRNA and by a dominant negative Akt variant. Accordingly, incubation of RPE cells with uPA elicits a time–dependent enhancement of MAPK and PI3K kinase activities as well as the transcriptional activation of Bcl–xL anti–apoptotic factor. Vice-versa, silencing of Bcl–xL expression prevents uPA protection from anoikis. In conclusion, the data show that ligand–engagement of uPAR promotes cell survival by activating Bcl–xL transcription through the MEK/ERK– and PI3K/Akt–dependent pathways.

Keywords: apoptosis, uPAR signaling, Bcl–xL
Urokinase receptor activation by a novel interaction between connecting peptide region of urokinase and alphavbeta5 integrin

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The serine protease urokinase (uPA) binds to the receptor (uPAR) through its growth factor domain (GFD, residues 1-49), affecting cell migration, adhesion, and growth. We present evidence that uPA can promote cytoskeletal rearrangements and directional cell migration in a GFD-independent manner, through a novel and specific interaction between an internal uPA domain coined “connecting peptide” (residues 132–158) and cell surface integrin avb5. Remarkably, a peptide corresponding to this region (CPp, residues 135–158) retains the ability to bind to avb5, eliciting cytoskeletal rearrangements and directing cell migration at a concentration as low as 1–10 pM. These effects are lost in cells not expressing uPAR, indicating that the uPAR is required for CPp-dependent signaling. Furthermore, the CPp/avb5 interaction enhances F–actin–enriched protrusions and cell migration induced by the well-established interaction between the uPAR–binding peptide (GFDp, residues 12–32) of uPA and uPAR. These results provide new insight into the function of uPA which, through individual domains, can engage two different surface receptors (uPAR and avb5 integrin), thus initiating and potentiating intracellular signaling and migration.

Keywords: uPA connecting peptide, uPAR, alphavbeta5
It has long been recognized that serine proteases initiate cellular changes. However it was not until the identification of the “protease activated receptor” (PAR) family in the 1990s that many of these changes could be explained at the molecular level. Using calcium flux and ERK activation assays and microscopy approaches we have shown that a recently identified serine protease signals via a member of the protease activated receptor family. Interestingly it also appears that cellular changes induced by this serine protease occur via a non–PAR mediated pathway. This result and our work, and that of others, on the cancer associated transmembrane protein SIMA135, suggest that there are non–PAR cell surface proteins which function to transduce extracellular serine protease initiated signals across the plasma membrane. This proposal is consistent with the plethora of known cell surface serine protease targets.

**Keywords:** serine protease, receptor, cell surface
The metastasome defines lynchpin uPAR-driven protein:protein interactions in epithelial cancer

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The urokinase plasminogen activator receptor (uPAR) is a key player in cancer metastasis as it can enhance proteolytic degradation of ECM barriers, activate nascent growth factors and activate a plethora of signal transduction pathways. In order to fully characterize how uPAR elicits such a multitude of effects, orthogonal proteomic approaches were employed based on co-immunoprecipitation of uPAR complexes from malignant ovarian cancer cells (OVCA 429). A cluster of proteins that specifically co-purified with anti-uPAR mAbs were identified by advanced proteomic techniques (i.e., 1D SDS/PAGE gels, biochip MALDI and multi-dimensional LC–MS/MS) and a number of proteins were validated using immunological approaches (e.g., cross-over immunoprecipitation pulldowns with Western blotting). Receptor uPAR–interacting proteins identified included a number of well known (and some novel) proteins. Of particular interest were a range of signal trasduction proteins and those previously associated in some way with the regulation of plasminogen activation on the cell surface (e.g., beta 6 integrin subunit, thrombospondin, enolase, alpha-V integrin, phospholipase C, LRP, TGF–beta receptor2 and annexin A2. The physical interaction between uPAR and ?v?6 has for the first time been definitively confirmed by proteomics and cross-over Western blotting approaches, as has interaction of TGF–?R2 in the complex involving uPAR. Studies are ongoing to determine the precise sites of interaction between the various players in the complex. We have also extended these proteomic studies to examine some functional consequences of these interactions and show that disruption of MAP kinase activation through antagonism of particular interactions and a redirecion of TGF–beta driven effects on cell invasion not suprisingly through manipulating cell–surface uPAR under- and/or over–expression. Collectively, our data demonstrates for the first time that uPAR may mediate its biological effects in cancer not through single interactions [1–3] but through the formation of a plethora of protein:protein interactions that involve cell–surface and intracellular proteins – a complex for discussion purposes we have colloquially termed the “metastasome”. 1. Degryse B, Resnati M, Czekay RP, Loskutoff DJ, Blasi F. Domain 2 of the urokinase receptor contains an integrin–interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. J Biol Chem. 2005; 280, 24792–803. 2. Bass R, Werner F, Odintsova E, Sugiura T, Berditchevski F, Ellis V. Regulation of urokinase receptor proteolytic function by the tetraspansin CD82. J Biol Chem. 2005: 280, 14811–8. 3. Kugler MC, Wei Y, Chapman HA. Urokinase receptor and integrin interactions. Curr Pharm Des. 2003; 9,1565–74.

Keywords: uPAR, proteomics, metastasome
Activation of Glu-, Lys- and mini-plasminogen by equimolar quantities of streptokinase 36 kDa fragment and effect of fibrin on this process

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Native streptokinase (Sk, 47 kDa) activates circulating plasminogen (Pg) and fibrin-bound plasminogen, but the activation rate of the last is higher. The ability of streptokinase to activate plasminogen in the absence of fibrin limits its therapeutic utility. Recombinant Sk without the 59 N-terminal amino acid residues activates Pg through fibrin-dependent mechanism. Limited proteolysis of 47 kDa Sk by plasmin or chymotrypsin coverts it to 36 kDa fragment (Sk36) lacking the 63 N-terminal and 34 C-terminal amino acid residues. The aim of this work was to study the role of fibrin in activation Pg by Sk36. It was investigated activation Glu-, Lys- and Val442–plasminogen (mini–Pg) by equimolar quantities of Sk36 and effect of fibrin on this process. Sk36 was isolated from chymotryptic digest of native Sk by Trisine–SDS PAGE. Activation Glu–, Lys– and mini–Pg were evaluated by measuring the amidolytic activity of generated active complexes with using a paranitroanilide substrate (S2251). Sk36 (50 nM) did not activate Glu–Pg (50 nM) during 1 hour. Lys–(50 nM) and mini–Pg (50 nM) was activated by equimolar quantities of Sk36 during the same time. Activation of the Lys– and mini–Pg began after 20 and 10 min lag–period. The cleavage rate of S2251 by formed active complexes was 0.007 and 0.041 o.u./min at 405 nm for Lys– and mini–Pg respectively. Fast activation rate of mini– Pg by Sk36 indicates that the activator binding site, necessary for the active centre formation, is exposed in the mini–Pg molecule. In the presence of fibrin (1,2 mkM) activation of the Glu–Pg (50 nM) by Sk36 (50 nM) began after 40 min lag–period. Fibrin decreased the lag–period of Lys–Pg activation reaction to 2-fold, whereas it did not change on the mini–Pg activation rate. The cleavage rate of S2251 by formed active complex in the fibrin presence was 0.015, 0.042 and 0.043 o.u./min for Glu–, Lys– and mini–Pg respectively. Thus fibrin effectively enhanced the activation rate of Glu– and Lys–Pg by Sk36 and did not change the activation rate mini–Pg one. It is supposed that on the fibrin surface are sites with the similar structure to N-terminal peptide of the native Sk. Interaction of the Glu– and Lys–Pg with this site results to exposure of Sk36 binding site in the zymogene and enhancement of active centre forming rate.

Keywords: streptokinase, plasminogen, fibrin
The Effects of Oral Contraceptives on Plasma - Studied by Proteomics.

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The use of oral contraceptives (OC) is known to influence a variety of biochemical components in human blood. Epidemiological studies have demonstrated that OC increase risk of thrombotic diseases. The risk of venous thrombosis is higher for OC preparations containing desogestrel or gestodene (third–generation progestogens) than for those containing levonorgestrel (second–generation progestogen). Studies of the effect of OC have shown that the concentration of pro–coagulatory and pro–fibrinolytic variables such as prothrombin and plasminogen is increased while the concentration of anticoagulatory and antifibrinolytic variables such as antithrombin and plasminogen activator inhibitor–1 is reduced by treatment with OC. We have used proteomics approach based on high–resolution 2–dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) to study the influence of second and third–generation OC on plasma protein levels and modifications. Plasma samples from two groups of women (13 individuals in each group), one treated with second–generation OC and the other with third–generation OC were studied before initiation of the treatment and after 12 months use of OC. We have created a reference 2DGE proteome map of Sypro Ruby stained plasma proteins based on 6 IPG gel gradients (4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7, 4.0–7.0 and 6.0–9.0). The gel images from the two patient groups have been analysed by ProteoMiner image analysis software. Protein markers were selected for MS analysis and have been cut out from non fixed wet preparative gels. The results show a number of individual differences between patients, but also clear differences that can be linked to the use of OCs.

Keywords: oral contraceptives, plasma, proteomics
Apoptosis is a process by which cells undergo programmed cell suicide. In principal, cell proliferation and apoptosis play a major role in maintaining homeostasis. However, in many instances the rate of apoptosis either exceeds or is much reduced than that of proliferation, thereby becoming implicated in various disease and clinical states. Apoptosis occurs as the outcome of various signalling pathways within the cell. It is known that proteases are involved within these pathways, particularly in the cleavage of zymogens, necessary for the activation, or maturation of apoptotic enzymes. Unregulated apoptosis, either as increased or decreased rates, can be due to a disruption in the balance and control of both intra- and intercellular proteolytic activities. Increasingly research has implicated serine proteases within the processes of apoptosis. We endeavoured to identify novel serine proteases through the application of a library of ‘in-house’ and commercially available inhibitors. Verification of apoptotic death was established using several characteristic endpoints, including measurement of cell viability, caspase-3 activation, PARP cleavage and the presence of phosphidylserine on the outer leaflet of the plasma membrane. We believe we have identified a chymotrypsin–like protease whose function may contribute to promoting cell viability. Quantitative cell viability measurements were obtained through application of a standard MTT assay. Caspase–3 activity was measured fluorogenically utilising the caspase–3 substrate Ac–DEVD–7–amino methylcoumarin. In addition, western blotting techniques were used to identify caspase–3 activation and PARP cleavage. Translocation of phosphidylserine to the outer leaflet of the plasma membrane was visualised using Annexin V staining and fluorescent microscopy. This peptide screening process led to the detection of a chymotrypsin–like protease targeted by tetramethylrhodamine–phenylalanine–diphenyl phosphonate. Incubation with this labelled peptide induced a reduction in cell viability to 9% of control values in both HeLa and U251 cell–lines. Decreased HeLa cell viability was instigated following 3 hour incubation with the inhibitor, whereas interesting, U251 cells demonstrated resilience, retaining 100% viability until 12–18 hours post–incubation. Furthermore, correlating with reduced cell viability, a time–dependent increase in caspase–3 activity was observed, as was PARP cleavage. In agreement with these findings, phosphidylserine translocation was detected, commencing 3 hours post–incubation in HeLa cells. Uptake and localisation of tetramethylrhodamine–phenylalanine–diphenyl phosphonate in cells has been observed through confocal microscopy, with early detection showing potential endosomal trafficking/location. Attempts have been made to identify this protease. Although molecular weight has been established as being approximately between 52 and 60 kDa, sequencing the protease has proved relatively problematic. To date, immunoprecipitation methods have been unsuccessful, potentially due to the close coupling of the tetramethylrhodamine label to the phenylalanine amino group. Alternative identification methods are currently being followed utilizing a biotin–labelled phenylalanine diphenyl phosphonate which we have demonstrated binds to the active site of our target protease. In summary, a chymotrypsin–like protease involved in apoptotic processing has been identified through the utilisation of various labelled diphenyl phosphonates. Isolation of this protease and subsequent sequencing will confirm the discovery of a novel apoptotic protease or alternatively, the novel application of peptide–labelling to a previously documented protease. (494 words)

**Keywords:** Apoptosis, Caspase, Protease
Activity Based Protein Profiling of HT-1080 Variants Identifies uPA Activation as a Key Regulator of Tumor Cell Intravasation

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Entry of malignant cells into the vasculature, i.e. intravasation, requires proteolytic remodeling of the extracellular matrix so that tumor cells may pass through the local stroma and penetrate the vessel wall. The circulatory system then provides a means of transporting tumor cells to distant sites where they establish metastatic lesions. We utilized activity based protein profiling (ABPP) to compare the active serine hydrolase repertoire of high–intravasating (HT-hi/diss) and low–intravasating (HT-lo/diss) variants of the human fibrosarcoma HT–1080 cell line, recently isolated in our laboratory, to determine which enzyme(s) play a role in intravasation. ABPP is a chemical proteomic technique that uses active site directed probes linked to a reporter group to measure the activity levels of individual enzymes of a given class in a complex mixture of proteins. ABPP combined with mass spectroscopy can identify enzymes that have altered activity levels between experimental groups, such as metastatic and non–metastatic tumor cells. Since proteins are detected based on activity rather than abundance, ABPP can distinguish between active enzymes and their inactive zymogen or their inhibitor bound forms, thereby providing an advantage compared to other methods of profiling. ABPP analysis of HT-hi/diss and HT-lo/diss cells revealed multiple serine hydrolases with altered activity between the two variants, with the largest difference being the activity of urokinase plasminogen activator (uPA). While the level of uPA expression has been implicated in the metastatic process, we show that activation of uPA is a key step due to the fact that uPA protein levels in the two variants are the same. The failure to activate uPA may play a large role in the inability of HT-lo/diss cells to intravasate and metastasize, while the active uPA in HT-hi/diss cells likely contributes to the intravasation ability of these cells. To determine the biological significance of this difference in uPA activity, we used the HT-lo/diss and HT–hi/diss variants in a chick–embryo chorioallantoic membrane (CAM) assay to quantitatively measure intravasation and metastasis in vivo. We also took advantage of the activity based nature of the ABPP system to screen small molecule inhibitors of uPA for their specificity and efficacy, to determine the optimal uPA inhibitor for use in the CAM assay. Inhibition of uPA activity with natural (PAI–1) or synthetic (amiloride) inhibitors significantly diminished HT–hi/diss intravasation and metastasis in vivo. Additionally, treatment of HT–lo/diss tumors with exogenous active uPA increased the number of intravasated cells in vivo 2–3 fold. These results indicate that active uPA promotes tumor cell intravasation and that uPA activation appears to be a key step in tumor progression. These experiments highlight the efficiency of ABPP analysis in the identification of specific proteases that regulate tumor progression. The combination of ABPP analysis with the chick embryo CAM assay has proven to be a powerful and rapid technique for identifying potential regulators of tumor dissemination and for modulating their activities in vivo to confirm their effect on the metastatic process.

Keywords: Activity Based Proteomics, Intravasation, uPA
**Serpins**

**ID:** 53

**Effect of alpha-2-antiplasmin on activation of plasminogen by different activators and following fibrinolysis**

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Activators of plasminogen (Pg), such as tissue plasminogen activator (t–PA) and streptokinase (SK), bring to formation highly specific serine protease – plasmin (Pm). Activation of Pg by SK occurs in solution, the presence of fibrin enhances the activation rate, but SK doesn’t bind with fibrin. In contrast, necessary condition of rapid conversion Pg by t–PA is binding these proteins with specific sites on fibrin. The half-time of free plasmin in the blood estimated to be approximately 0,1 s, on the fibrin surface – 100 s. This fact may be explained by protection of enzyme from inactivation by alpha–2–antiplasmin. The main function of alpha–2–antiplasmin is inhibition of plasmin releasing from fibrin surface after it degradation and protection of plasma proteins from nonspecific proteolysis. Effect of alpha–2–antiplasmin within a fibrin clot on t–PA–mediated activation Pg and following lysis remain unknown. We compared the inhibition SK– or t–PA–induced clot lysis by alpha–2–antiplasmin in a purified system using of the turbidimetric method. Fibrin clot formed 200 ?g was hydrolyzed by 3 ?g Pm during 3–4 min. The addition to mixture the same amount of Glu–Pg and catalytic amount of SK (molar ratio 10:1) increased fibrin lysis by 10%. Pg activation by SK in the absence of fibrin occurred during 1 hour. We suggested that enzyme concentrates on a fibrin surface and transition into conformation which more appropriate for activation. The addition 1,5–fold molar excess of alpha–2–antiplasmin to Pm inhibited lysis completely. If fibrin polymerization occurred at the molar ratio Pg:SK:alpha–2–antiplasmin 1:0,1:1,5 clot lysis was equal the same in the absence of inhibitor or by Pm. We consider that all Pm, which formed, is on the fibrin surface at activation Pg by catalytic amount of SK thus it protects from inhibition by alpha–2–antiplasmin. Maximal lysis rate was demonstrated at 30 IU t–PA/ 1 ?g Pg and made 7 min. The addition equimolar amount of alpha–2–antiplasmin increased fibrin lysis by 10–fold. These results show that alpha–2–antiplasmin appear is strong inhibitor of physiological fibrinolysis. Inhibition of may occur at the level of plasminogen activation by tissue activator on fibrin surface.

**Keywords:** alpha–2–antiplasmin, activation of plasminogen, fibrin
A Novel ELISA for Mouse Activated Protein C in Plasma Based on Interspecies Inhibition by Human Protein C Inhibitor

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Purpose: Protein C is the zymogen of activated protein C (APC). Protein C is activated in vivo by the thrombin–thrombomodulin complex and this activation is enhanced by the endothelial cell protein C receptor. The Protein C pathway plays a crucial role in the regulation of thrombosis and inflammation. To elucidate mechanisms of action of APC researchers presently use transgenic or gene deletion murine models. To correlate observations in these murine models with the APC levels, there is a need for a sensitive and specific assay for circulating murine APC in plasma. We developed an immunological assay to measure the physiologic and pharmacologic levels of circulating murine APC.

Methods: The sandwich ELISA uses a rabbit polyclonal anti-murine protein C antibody capture antibody and human protein C inhibitor (PCI) as a detection reagent, because human PCI forms a 1:1 stable complex with mouse APC and the mouse plasma lacks PCI. The amount of complexed PCI is detected with an anti-PCI monoclonal antibody which is then detected using biotinylated anti-mouse IgG and streptavidin conjugated to horseradish peroxidase.

Results: The assay measured active APC in mouse plasma, since only APC with a free active site is complexed with human PCI. The capturing antibody was generated from recombinant mouse protein C and it was monospecific in mouse plasma. The detecting antibodies were also specific because no mouse PCI is present in mouse plasma and the monoclonal antibodies are raised using human PCI. Plasma was collected in citrate anticoagulant containing benzamidine–HCl to prevent the inhibition of APC in vitro by mouse plasma protease inhibitors. The assay showed improved sensitivity versus enzyme immunocapture assays (ECA’s) commonly used to detect human APC. After each component of the assay was optimized, the assay was sensitive to low amounts of murine APC (≥30 pg) and was linear over a wide range (30–320 pg APC per well). The design of this assay makes it convenient for high-throughput screening of multiple samples because it substantially shortens the time compared to assays developed for circulating human APC. It is well suited for measuring the endogenous generation of APC by thrombin infusion. The basal level of APC in pooled murine plasma was 49 pM, and circulating APC levels were considerable increased when thrombin was infused into mice. Measuring circulating APC in plasma will be useful in understanding the mechanisms that are involved in APC generation in murine models of disease. The assay can be also useful to determine the baseline levels of APC in plasmas of mice with genetic alterations that can affect the protein C pathway in vivo.

Conclusions: In summary, we have described an immunological assay for determining the concentration of mouse circulating APC in plasma which is 49 pM in pooled mouse plasma. This assay specifically measures murine APC in plasma over a physiological range of concentrations, has low backgrounds, and is suitable for screening multiple samples. This method for mouse APC represents a marked improvement over published assays for the detection of human APC.

Keywords: Activated Protein C, Protein C Inhibitor, Mouse
A structural basis for differential cell signaling initiated by PAI-1 and PAI-2: Implications for Metastatic Potential

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PAI-1 and PAI-2 (serpin E1 and B2, respectively) are both potent inhibitors of cell surface urokinase plasminogen activator (uPA). However, tumour expression of PAI-1 strongly correlates with poor patient prognosis, while PAI-2 generally correlates with good patient prognosis. Currently, there are no adequate biochemical/functional data to explain this discrepancy. One theory for the tumour-pro-moting effects of PAI-1 is based on the signaling events initiated by inhibition of cell surface uPA. The inhibition of uPA by PAI-1 is known to reveal a cryptic high affinity site within the PAI-1 moiety that binds to members of the low density lipoprotein receptor (LDLR) endocytosis receptor family. For example, binding of this high affinity site in PAI-1 to the very low density lipoprotein receptor (VLDLr) sustains an otherwise transient ERK phosphorylation initiated by binding of uPA to uPAR. These inter-actions and subsequent signaling events have been shown to promote both cell migration and prolif-eration. Through biochemical analysis we have shown that unlike PAI-1, the PAI-2 moiety of uPA:PAI-2 does not contain a high affinity binding site for LDLR family molecules, although the uPA:PAI-2 complex is still efficiently endocytosed by these receptors. Hence, upon inhibition of uPA, PAI-2 may not induce the signaling events associated with PAI-1 inhibition, providing a potential functional basis for the differing prognoses associated with PAI-1/PAI-2 tumor expression. Therefore, we compared the signaling responses initiated by uPA, uPA:PAI-1, uPA:PAI-1R76E (a PAI-1 mutant that lacks the high affinity LDLR binding site) and uPA:PAI-2 in MCF-7 human breast carcinoma cells. The transient ERK phosphorylation stimulated by the binding of uPA to uPAR was sustained by the addition of uPA:PAI-1 but not by the addition of uPA:PAI-2 or uPA:PAI-1R76E. The corresponding cell prolifera-tion associated with uPA:PAI-1 binding to uPAR and VLDLr was not observed following the addition of uPA:PAI-2 or uPA:PAI-1R76E. Confirming the functional requirement for LDLR molecules, the stimulat-ory effects of uPA:PAI-1 were not observed in the presence of RAP. Furthermore, we have also demonstrated that unlike PAI-1, PAI-2 is not able to bind LRP independently of uPA. Therefore, the Jak/Stat pathway activation associated with PAI-1 binding to LRP, which also stimulates migration of cancer cells, would not be expected to be initiated by PAI-2. These data therefore present a possible mechanism by which PAI-2 is able to clear cell surface uPA, and hence proteolytic activity, via LDLRs without the cell signaling events and increased metastatic potential associated with high PAI-1. This may partially explain why high tumour levels of PAI-1 are correlated with a poor prognosis, whereas high tumour expression of PAI-2 correlates with a good prognosis.

Keywords: PAI-1, PAI-2, Cell Signaling
Assessment of known/potential binding sites in the PAI-2 CD-loop for interaction with annexin II and endocytosis receptors

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Dysregulation of the urokinase (uPA) system is a well established element of cancer invasion and metastasis. The two efficient uPA inhibitors, serine protease inhibitors (serpins) PAI-1 and PAI-2 are the key regulators of the system, although they are structurally and functionally distinct. Furthermore, their role in cancer invasion and metastasis is contradictory: while high uPA/PAI-1 expression is a prognostic marker of progression in early breast cancer, high tumour PAI-2 expression is generally related to a favorable overall survival. A possible explanation for such behaviour may be different cell signalling following clearance of PAI-1/uPA/uPAR and PAI-2/uPA/uPAR complexes from the cell surface. Both are cleared through a uPA moiety that interacts with endocytosis receptors. However, PAI-1 has additional endocytosis receptor binding sites which further potentiate downstream signalling via these endocytosis receptors, stimulating cell proliferation and migration. These sites are absent in PAI-2 and the signalling effects have not been demonstrated for the serpin. Another explanation may lie within a unique PAI-2 structure called the CD-loop which connects helices C and D of the serpin. There are three known protein binding sites within the CD-loop: (i) Cys 79 is responsible for binding of PAI-2 to vitronectin in vitro in a redox-sensitive manner, (ii) residues 73–76 bind to retinoblastoma protein and (iii) the glutamine residues 83, 84 and 86 can be crosslinked by trophoblast transglutaminase. Furthermore, our recent observations show that PAI-2 is capable of binding via the CD-loop to the cell surface annexin II. Therefore, we are initially testing the known/potential protein binding sites in the PAI-2 CD-loop to identify annexin II binding site(s) and to test their impact on PAI-2 endocytosis. We created and purified four PAI-2 mutants: (i) Cys 79 Arg substitution, (ii) deletion of amino acids 73–76 (PENF), (iii) deletion of amino acids 83–86 (QQIQ) and (iv) deletion of amino acid residues 97–99 (QAQ), a potential site of transglutamination and a potential secondary structure site. We have also purified wt PAI-2 and PAI-2 ?CD-loop mutant (amino acid residues 66–98 deleted, i.e. entire CD-loop). The mutants were created using the ExSite PCR-based site-directed mutagenesis (Stratagene) of his-tagged wt PAI-2 in pET15b as template. All six PAI-2 proteins were expressed in E.coli and purified via metal affinity and ion-exchange chromatography techniques. The uPA inhibitory activity of the expressed proteins was verified by SDS–PAGE analysis after incubation with uPA. Our current plans are to compare the six PAI-2 proteins for their ability to bind annexin II in vitro by using pull down experiments and surface plasmon resonance analysis. Furthermore, we will assay colocalisation of PAI-2/annexin II and PAI-2 endocytosis in cultured cancer cell lines with different abilities to express components of the uPA system, the endocytosis receptors and annexin II.

Keywords: PAI-2, CD-loop, annexin II
Phosphatidylserine and oxidized phosphatidylethanolamine interact with Protein C Inhibitor (PCI) and modify its activity

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Protein C inhibitor (PCI) is a non-specific, heparin-binding serine protease inhibitor (serpin). Target proteases inhibited by PCI include activated protein C (aPC), thrombin, urokinase (uPA), acrosin, plasma and tissue kallikreins. Heparin enhances the interaction of PCI with most proteases. Nishioka et al. (1998) have shown that PCI also binds to phosphatidylethanolamine (PE), and that PCI inhibits phospholipid-bound aPC efficiently. It was our aim to further analyze the interaction of PCI with different phospholipids, and to study its biological relevance. We can show that PCI binds to phosphatidylserine (PS; oxidized and unoxidized) and to oxidized PE, both immobilized on a surface (microtiter plate) and in fluid phase (native PAGE). Binding to other phospholipids was observed only when they were immobilized. Binding of PCI to oxidized PE and to PS was competed by heparin, but not by retinoic acid, which has been shown to bind PCI (Jerabek et al., 2001), or the PS-binding protein annexin V. Binding of PCI to PS and oxidized PE was Ca++-independent. PS (oxidized as well as unoxidized) and oxidized PE stimulated the interaction of aPC with PCI. This effect was strongly dependent on Ca++, indicating that binding of both, aPC (Ca++-dependent) and PCI (Ca++-independent), to PS or oxidized PE is necessary. In the absence of Ca++, oxidized PE and PS interfered in a dose-dependent manner with the interaction of aPC with PCI. This effect was antagonized by heparin. Similar to heparin (Ecke et al., 1992), PS and oxidized PE interfered with the inhibition of tissue kallikrein by PCI. These data suggest that certain phospholipids could be a more general substitute for heparin as far as its effect on the inhibitory activity of serpins is concerned. However, the effect of PS and oxidized PE seems to be specific for PCI, since it was not seen with antithrombin III, another heparin-binding serpin. In conclusion, our data indicate that in addition to heparin and glycosaminoglycans also negatively charged phospholipids can modulate the activity of PCI. Binding to these phospholipids seems to involve the heparin-binding site of PCI. Exposure of oxidized PE and/or PS may be important for the local accumulation of PCI and for the regulation of its activity in vivo. In fact, preliminary immunohistochemical data show that PCI is localized at sites where PS and/or oxidized phospholipids are exposed (e.g. on the syncytiotrophoblast of the placenta, in atherosclerotic plaques).

Keywords: Protein C Inhibitor, Phospholipids, activated Protein C
**Structural Studies**

**ID:** 343

**Interactions between Urokinase-Type Plasminogen Activator and Its Receptor Defined by the X-ray Crystallography**

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The urokinase plasminogen activator receptor (uPAR, CD87, Mo3), in close association with urokinase (uPA)/plasminogen components, plays a pivotal role in diverse (patho-)physiological processes including angiogenesis, wound healing, and cancer metastasis. The wealth of biochemical studies point towards the presumed conformational changes accompanying uPA–uPAR engagement, but the structural data addressing this phenomenon are scarce. The objective of this study was to provide structural basis for biochemical data describing the nature of uPA/uPAR interactions as well as define conformational changes of the receptor upon uPA binding. To this end, we crystallized and solved X-ray structures of the soluble form of uPAR (suPAR) in complex with its ligand(s). Our results allow detailed description of intermolecular interactions between uPAR and uPA and moreover, through comparison to a previously published structure of suPAR bound with a small peptide inhibitor, reveal spatial rearrangements of the receptor induced by the uPA binding. Observed structural changes comprise overall realignment of entire domains of suPAR as well as more subtle readjustments of the regions previously implicated in chemotaxis and integrin interactions. Additionally, we solved the 1.9 Å crystal structure of the free ATF. This structure enabled the relative orientation of the individual ATF domains and the inter–domain contacts to be analyzed for the first time, and comparison of the receptor bound and free ATF allows us to draw conclusions linking the inter–domain contacts within ATF to physiological processes described in the literature.

**Keywords:** urokinase receptor, protein–protein interactions, X–ray crystallography
Biochemical characterization of derivatives of MA-T12D11, a TAFI neutralizing antibody

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Introduction: TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded fibrin degradation products thereby abolishing their cofactor function in the activation of plasminogen by t-PA. A previous study identified 14 monoclonal antibodies, raised against human TAFI, that exert a TAFI inhibitory effect by impairment of the activation of TAFI into TAFIa by thrombin/thrombomodulin. Surprisingly, only three of these antibodies could also hamper substantially the activation by plasmin even though activation by plasmin and thrombin involves the same cleavage site, i.e. Arg92. Epitope analysis identified Gly66 as the major residue for the neutralizing antibodies that inhibit exclusively the activation of TAFI by thrombin/thrombomodulin, and this group is represented by MA-T12D11.

Objective: To study the biochemical properties of MA-T12D11 derivatives in order to gain more insights in the working mechanism of MA-T12D11.

Methods and results: Two antibody derivatives of MA-T12D11 were generated. Firstly, a Fab-T12D11 fragment was produced by papain digestion of MA-T12D11, followed by protein A purification. Secondly, a single-chain variable fragment (scFv) of MA-T12D11 was cloned. Therefore, cDNA was isolated from MA-T12D11 producing hybridomas, and VH and VL regions were amplified with the appropriate primers and assembled using a DNA linker segment (encoding (Gly4Ser)3). As expected, sequencing of this scFv revealed the typical presence of CDR regions. Surprisingly, affinity constants of Fab-T12D11 and scFv-T12D11 for TAFI-AT (KA = 2.8 ± 1.0 x 10e9 1/M and KA = 1.7 ± 0.7 x 10e9 1/M, respectively) were only 2- to 3-fold lower compared to that of MA-T12D11 (KA = 5.4 ± 1.5 x 10e9 1/M). Evaluation of the dose-response curves of the Fab and scFv fragment on the inhibition of TAFI activation revealed a shift towards slightly higher concentrations. This is in line with the small decrease in affinity and eventually resulted in a similar maximum effect as the corresponding MA-T12D11 (i.e. 98 ± 1%, 99 ± 1% and 91 ± 3% TAFIa inhibition using a 8-fold molar excess of MA-T12D11, Fab-T12D11 and scFv-T12D11, respectively). Evaluation of the profibrinolytic effect of MA-T12D11 derivatives was tested in a clot lysis assay and compared to potato tuber carboxypeptidase inhibitor (PTCI), a known inhibitor of TAFIa. In the absence of an inhibitor, the clot lysis time was 120 min. Preincubation of plasma with PTCI (45-fold molar excess) shortened the clot lysis time to 32 min. Preincubation with MA-T12D11(8-fold molar excess) and Fab-T12D11(8-fold molar excess) shortened the clot lysis time to 25 min and 26 min, respectively. The effect of scFv-T12D11 in the clot lysis assay will be tested.

Conclusion: Characterization of MA-T12D11 derivatives reveals that these fragments have similar affinity constants as their parental MA and have a similar mode of action on TAFI activation by thrombin/thrombomodulin. The availability of the cloned scFv and its sequence also allows to further explore the paratope characteristics contributing to the functional effects of MA-T12D11 and serve as an excellent starting point for structure-based drug design.

Keywords: TAFI, antibody fragments, fibrinolysis
Fibrinolytic efficacy of Amediplase, Tenecteplase and scu-PA in different external plasma clot lysis models. Sensitivity to the inhibitory action of thrombin activatable fibrinolysis inhibitor (TAFI).

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During thrombolytic therapy the plasminogen activator (PA) reaches the thrombus after its formation and lysis occurs in a sharp zone on the periphery of the thrombus (external lysis). TAFI is activated by thrombin and more efficiently by the thrombin/thrombomodulin (TM) complex. TAFIa interferes with the localisation of the plasminogen activation on the surface of fibrin by removing carboxy-terminal lysine residues from partially-degraded fibrin and is therefore considered to be one of the inhibitors regulating the fibrinolytic system. Here, we compared the lytic efficacy of three PAs (Tenecteplase, TNK–tPA; Amediplase, k2t–uPA and scu–PA) in external plasma clot lysis and studied their sensitivity to TAFI. In the first model, plasma clots were formed by incubating (30 min, 37 degrees) plasma, supplemented with trace amounts of FITC-labelled fibrinogen, with calcium (20 mM) and thrombin (1 NIH unit/ml). The clots were then immersed in plasma supplemented with the PA (0–10 microg/ml) and with and without a direct thrombin inhibitor (hirudin, 20 ATU/ml). The clots were incubated under stirring at 37 degrees and the extent of lysis was determined at regular intervals by measuring the release of FITC–labelled degradation products. The extent of lysis was plotted against the incubation time to calculate the half-maximal lysis time (LT). In the second model plasma clots were formed in a similar way (20 mM calcium, 0.3 NIH units/ml thrombin) but now at room temperature and with or without potato carboxypeptidase inhibitor (PCI, 30 microg/ml), a TAFIa inhibitor. Defibrinated plasma was prepared by incubation with batroxobin, which is unable to activate TAFI. Clots were immersed in defibrinated plasma supplemented with TM (0.1 unit/ml), calcium (20 mM), the PA and with or without PCI. The clots were incubated at room temperature and the lysis was determined as described above. In the first model, k2t–uPA was less active than TNK–tPA at low concentrations but slightly more active at high concentrations, whereas scu–PA was less active than both TNK–tPA and k2t–uPA at all concentrations tested. Addition of hirudin had no effect on the LTs suggesting that only small amounts of thrombin were generated, too low for substantial TAFI activation. In the second model, particularly in the presence of PCI, the PAs showed similar activities as in the first model. In the absence of PCI, the PAs were less active, indicating that TAFIa inhibited all PAs. The inhibitory effect was high at low PA concentrations and negligible in the therapeutic range (TNK–tPA, k2t–uPA). Below 1 microg/ml, k2t–uPA lost its fibrinolytic potential more rapidly than TNK–tPA and was more sensitive to TAFIa. In conclusion, k2t–uPA was more active than TNK–tPA and scu–PA at therapeutic concentrations under all conditions tested. Though the role of TAFI during successful thrombolytic therapy seems small, TAFI might affect the lysis rates when the local PA concentrations are sub–optimal.

Keywords: TAFI, plasminogen activators, fibrinolysis
The Thr325Ile polymorphism and intrinsic enzymatic activity of proCPU (TAFI): New pitfalls interfering with straightforward proCPU activity measurement in plasma.

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To measure plasma proCPU concentrations different methods have been developed based on two distinct principles: antigen determination and measurement of CPU activity after quantitative conversion of the proenzyme to its active form by addition of thrombin–thrombomodulin. Both types of assays deal with difficulties and pitfalls compromising straightforward measurement in a routine laboratory setting (reviewed in Willemse et al. Clin Chem 2006; 52, 30–36). By comparing a recently described kinetic proCPU assay and an HPLC-assisted endpoint proCPU assay, we investigated the influence of the Thr325Ile polymorphism on accurate proCPU determination. We found that the difference in stability between the two isoforms becomes a very significant interfering factor when (i) activation of the proenzyme is performed at 37°C or when (ii) substrate is not used in high excess and/or (iii) the incubation interval used is too long. In plasma two basic carboxypeptidases are known: carboxypeptidase N (CPN) and procarboxypeptidase U (proCPU). It is stated in literature that the basic carboxypeptidase activity in plasma is solely attributable to the constitutively active CPN. In our screening for more selective CPN and CPU substrates we found that proCPU shows intrinsic enzymatic activity depending on the substrate used and can therefore interfere with plasma CPN activity measurement and hence with proCPU determination since CPN activity is used as blank for proCPU determination. This interference was neglectable when Bz–Ala–Arg was used but becomes very significant using Bz–Met–Arg (up to 20%) so that newly introduced substrates should be first screened for enzymatic activity toward proCPU before they are used in e.g. commercial kits. The intrinsic enzymatic activity of proCPU can be inhibited with frequently used CPU inhibitors. Where PTCI is a 100-fold more potent inhibitor for CPU than for proCPU, GEMSA inhibits proCPU one order of magnitude better than CPU. In conclusion, we describe that both the polymorphism at position 325 and the intrinsic enzymatic activity of proCPU can have a profound effect on the determination of proCPU in plasma. This should be kept in mind in both the development and usage of proCPU activity based assays.

**Keywords:** TAFI, polymorphisms, assays

* selected for oral presentation
An increased risk of ischemic stroke is related to activated TAFI levels but not to genetic variation

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Background and purpose: Thrombin activatable fibrinolysis inhibitor (TAFI) has been described as a potent inhibitor of tissue-plasminogen activator induced fibrinolysis. The relevance of TAFI in ischemic stroke is however unclear. Previous studies indicate that plasma levels of TAFI are influenced by genetic factors. Aim of the present study was to investigate genetic variation at the TAFI locus and plasma levels of TAFI in ischemic stroke and its etiological subtypes.

Material and methods: The material comprises the Sahlgrenska Academy Study of Ischemic Stroke (SAHLSIS), which is a study from Western Sweden with 600 consecutive ischemic stroke cases below 70 years of age and 600 controls matched for age, sex and geographical region. Stroke subtype was defined by Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria. Among stroke cases, blood sampling was performed within 10 days of the event and at three-month follow-up. Plasma levels of nonactivated TAFI and released activation peptide (AP) were measured by two novel genotype-independent sandwich-type ELISAs. Based on HapMap data, seven single nucleotide polymorphisms (SNPs) were chosen to tag the TAFI locus. In addition, we genotyped four previously studied SNPs (G-438A, Ala147Thr, Thr325Ile and C1542G). SNPs were typed by TaqMan assays.

Results: Plasma levels of released AP as well as intact TAFI were significantly higher in overall ischemic stroke compared to controls, both in the acute phase and at three months follow-up (p<0.001 for all). After adjustment for traditional risk factors, the odds ratio (OR) for one standard deviation increase in released AP at follow-up was 2.22 (95%CI 1.89–2.61). The corresponding OR for intact TAFI at follow-up was 1.21 (95%CI 1.06–1.38). Acute levels showed a similar pattern. Furthermore, all stroke subtypes showed independent associations to released AP at both time points; ORs for follow-up levels were 2.77 (95%CI 1.86–4.10) for large vessel disease (LVD); 2.21 (95%CI 1.66–2.96) for small vessel disease; 2.46 (95%CI 1.82–3.33) for cardioembolic stroke and 2.27 (95%CI 1.80–2.86) for cryptogenic stroke. Intact TAFI showed independent association to LVD only: OR 1.63; 95%CI 1–19–2.21. Seven SNPs were required to identify 11 haplotypes with estimated frequencies above 1%, explaining 92% of the chromosomes. For 9 out of 11 SNPs, TAFI levels differed by genotype (p<0.01), in most comparisons this was observed both for released AP and for nonactivated TAFI. However, neither TAFI genotypes nor haplotypes showed a significant association to overall ischemic stroke.

Conclusion: Plasma TAFI is elevated in ischemic stroke compared to controls, and this difference is more pronounced for released AP than for nonactivated TAFI. With regard to released AP this association was observed for all etiological subtypes and the strongest association was seen for LVD stroke. This indicates that an increased risk of ischemic stroke is related to functional plasma TAFI levels. In contrast, no association was found between ischemic stroke and genetic variants.

Keywords: ischemic stroke, TAFI, genetics

* selected for oral presentation
A stable TAFIa variant as a tool to study TAFIa destabilization

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Introduction: Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFIa) plays a pivotal role in fibrinolysis and inflammation. The activity of TAFIa is limited by its instability which is highly temperature dependent. It was previously shown that the decay of TAFIa activity is associated with a substantial decrease in intrinsic fluorescence, implying that a conformational change accounts for the TAFIa instability. Moreover, detailed analysis of the fluorescence tracings revealed that there are two transitions (k₁ 0.5 min⁻¹ and k₂ 0.064 min⁻¹) during the conformational change from TAFIa to TAFIai. To date, the structural basis of TAFIa destabilization is still unclear. Recently, we constructed and characterized a stable TAFIa variant, i.e. TAFIa-C305-I325-I329, harbouring the stabilizing I325 residue of the naturally occurring polymorphism at position 325 and a S305C and T329I mutation. Based on activity measurements, this variant has a half-life of 70 ± 3 min at 37°C (vs 6.3 ± 0.3 min for TAFIa-wild type harbouring residue T325).

Objective: To determine the thermodynamic parameters and to monitor the change in intrinsic fluorescence during destabilization of TAFIa-wt, in comparison with that of TAFIa-C305-I325-I329, in order to gain more insight in the destabilization of TAFIa.

Methods & Results: To determine the thermodynamic parameters of TAFIa destabilization, TAFI-wt and TAFI-C305-I325-I329 were activated to TAFIa by thrombin/thrombomodulin and incubated for different time intervals at 25, 30, 33 and 37°C. The activation entropy (ΔS°) and activation enthalpy (ΔH°) for the process of TAFIa inactivation were obtained by fitting the rate constants (k) and temperature (T) as variables to the following equation describing the transition state theory: k = (kT/h)exp[(ΔS° - ΔH°/T)/R] (k = Boltzmann’s constant, h = Planck’s constant, R = molar gas constant). Activation entropy was 83 ± 18 cal/(K*mol) for TAFIa-C305-I325-I329 vs 58 ± 11 cal/(K*mol) for TAFIa-wt (n=3, P<0.05). Activation enthalpy was 48 ± 5 kcal/mol for TAFIa-C305-I325-I329 vs 39 ± 3 kcal/mol for TAFIa-wt (n=3, P<0.05). This corresponds with a 1.4-fold increased activation entropy and a 1.2-fold increased activation enthalpy for TAFIa-C305-I325-I329. To determine the two transitions that accompany the TAFIa to TAFIai conversion, the change in intrinsic fluorescence was monitored in a Quantamaster fluorimeter at 37°C (?ex 280 nm; ?em 340 nm). Decay constants for the first transition were 0.44 ± 0.21 min⁻¹ for TAFI-C305-I325-I329 vs 0.67 ± 0.23 min⁻¹ for TAFIa-wt (n=3, P=0.2). Decay constants for the second transition were 0.013 ± 0.003 min⁻¹ for TAFIa-C305-I325-I329 vs 0.075 ± 0.011 min⁻¹ for TAFIa-wt (n=3, P<0.001).

Conclusions: Determination of the thermodynamic parameters revealed an increased activation entropy for TAFIa-C305-I325-I329 suggesting that inactivation is more favourable. However, this is compensated by an increased activation enthalpy, which reflects the energy required to break noncovalent bonds. Detailed analysis of the fluorescence changes revealed that only the second transition in the TAFIa to TAFIai conversion is significantly slower in the stable TAFIa-C305-I325-I329 variant, suggesting that the functional instability of TAFIa is mainly associated with the second conformational transition step.

Keywords: TAFI, thermodynamic parameters, intrinsic fluorescence
MA-T18A8, the first monoclonal antibody directed towards a neoantigenic epitope in activated TAFI

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Introduction: On activation of Thrombin Activatable Fibrinolysis Inhibitor (TAFI), the activation peptide is released from the active enzyme TAFIa which exerts an antifibrinolytic effect. Previously, 42 MA were raised towards human TAFI. 19 of these MA (including MA-T12D11) revealed inhibitory properties whereas 23 MA (including MA-18A8) had no inhibitory effect. The MA-T12D11/MA-T18A8-HRP ELISA has a strongly increased reactivity for TAFI upon activation and also reacts significantly, although to a lower extent, with purified recombinant activation peptide. Gly66 was identified as the major residue for binding of MA-T12D11 to TAFI. Objective: To elucidate the binding region of MA-T18A8, a non-inhibitory MA raised towards human TAFI.

Methods and results: Western blotting experiments revealed that MA-T18A8 reacts with intact human TAFI-TI (i.e. TAFI-Thr147Ile325 isoform) and upon activation of TAFI with the released activation peptide. In addition, MA-T18A8 reacts with the recombinant human activation peptide. MA-T18A8 does also react with intact rat TAFI and with the released rat activation peptide but does not react with intact murine TAFI nor with the released murine activation peptide. In order to elucidate the epitope of MA-T18A8 a human/murine TAFI chimera TAFI–Hum67Mur, consisting of amino acids 1–67 of human TAFI and amino acids 68–401 of murine TAFI, was subjected to western blotting analysis revealing no reactivity of MA-T18A8 nor with intact TAFI–Hum67Mur neither with the released activation peptide. Surface Plasmon Resonance (SPR) experiments were performed to determine the affinities of MA-18A8. MA-T18A8 revealed no binding (KA<10e6 1/M) for intact human TAFI, murine TAFI and TAFI–Hum67Mur nor for activated murine TAFI and activated TAFI–Hum67Mur. In contrast with the western blotting experiments, MA-T18A8 does not bind to recombinant human activation peptide (KA<10e6 1/M). However, MA-T18A8 does bind to intact rat TAFI (KA=8.7±8.6 ?10e6 1/M). Upon activation of human TAFI and rat TAFI, affinity constants of 1.6±0.8 ?10e8 1/M and 4.7±1.8 ?10e7 1/M, respectively, were obtained.

Conclusion: From the results of the western blotting experiments, it can be deduced that a major part of the epitope of MA-T18A8 resides in the 68–92 region of the activation peptide and that most of the residues involved in the binding of human TAFI to MA-T18A8 are conserved in rat TAFI. This finding is only partly confirmed by SPR experiments which revealed a weak binding of intact rat TAFI, a moderate binding of human and rat TAFI upon activation and surprisingly no binding of the recombinant human activation peptide. It should be realized that in western blotting experiments, the protein is denatured whereas in SPR analysis the protein is used in its native form. Since activation of TAFI is a prerequisite for binding of MA-T18A8 to human TAFI and since MA-T18A8 does not bind to the recombinant activation peptide in a isolated non-denatured state, we conclude that the epitope of MA-T18A8 is neo-antigenic. Most likely, the major residues of this epitope reside in the activation peptide region, are exposed only after activation of TAFI or upon denaturation of the isolated activation peptide, and are mainly conserved between human and rat TAFI.

Keywords: TAFI, neoantigenic, MA-T18A8
Introduction: The proenzyme Thrombin Activatable Fibrinolysis Inhibitor (TAFI) can be activated to the active enzyme (TAFIa) by thrombin, plasmin or the thrombin/thrombomodulin complex. TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from fibrin thereby abolishing their cofactor function in the activation of plasminogen by t-PA. Although rat models are frequently used to study thrombolysis, monoclonal antibodies that react with rat TAFI are not available. Objective: The objective of the current study was to generate monoclonal antibodies towards rat TAFI and to assess their potential for application in in vivo studies.

Methods and results: TAFI knock-out mice were immunized with either recombinant rat TAFI or activated recombinant rat TAFI. The standard hybridoma technique yielded 5 monoclonal antibodies i.e. MA-RT13B2, MA-RT30D8, MA-RT36A3, MA-RT36B2 and MA-RT82F12. Although all five anti-rat TAFI monoclonal antibodies (MAs) cross-reacted with murine TAFI, only three antibodies i.e. MA-RT30D8, MA-RT36B2 and MA-RT82F12 cross-reacted with human TAFI in ELISA experiments. The inhibitory effect of the MAs on both TAFI activation and on TAFIa activity was tested using a chromogenic assay. None of these MAs was capable of blocking the activation of TAFI to TAFIa by thrombin/thrombomodulin. However, using a 16-fold molar excess of MA over TAFI, MA-RT13B2, MA-RT36A3 and MA-RT82F12 inhibited rat TAFIa activity with 77 ± 4 %, 51 ± 4 % and 21 ± 2 % and murine TAFIa activity with 80 ± 9 %, 57 ± 8 % and 33 ± 23 %, respectively. None of these MAs was capable of inhibiting human TAFIa activity. Clot lysis was performed in TAFI-depleted rat plasma and monitored at 405 nm. 20 nM thrombin, 5 nM thrombomodulin, 12.5 mM calcium chloride and 40 ng/ml tissue-type plasminogen activator was added to initiate clot formation and dissolution and revealed a clot lysis time of 7 ± 2 min. This clot lysis time increased to 37 ± 7 min when TAFI-depleted rat plasma was supplemented with 5 µg/ml recombinant rat TAFI and decreased to 6 ± 1 min in the presence of 25 µg/ml PTCI. A reduction of clot lysis time was observed upon addition of an 8-fold molar excess of inhibitory MAs over TAFI : MA-RT13B2 (22 ± 5 min), MA-RT36A3 (25 ± 8 min) and MA-RT82F12 (28 ± 9 min) whereas an 8-fold molar excess of the non-inhibitory MA-RT36B2 slightly increased clot lysis time up to 48 ± 16 min.

Conclusions: The current study describes the generation and characterization of 5 MAs towards rat TAFI and murine TAFI. Three out of five MAs were capable to inhibit rat and murine TAFIa activity directly and to partially reduce clot lysis time in vitro. Surprisingly, MA-RT36B2 increased clot lysis time, suggesting a stabilizing effect of the MA on rat TAFIa activity. The MAs are promising tools for in vivo investigation of the role of TAFI in rat models.

Keywords: rat TAFI, monoclonal antibody, inhibition
Binding of TAFI to collagen: a role for TAFI in the regulation of platelet adhesion?

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Thrombin-activatable fibrinolysis inhibitor (TAFI) forms a link between the blood coagulation and fibrinolytic systems. TAFI is activated by thrombin and once activated, it attenuates the tissue-type plasminogen activator dependent plasmin formation, hence slowing down clot dissolution. In addition, more and more data support a role for TAFI in other systems, such as inflammation and angiogenesis, and we recently observed that TAFI knockout mice have a delayed and impaired wound healing. The exact mechanism by which TAFI influences the process of wound healing is, however, unknown. For this reason, we studied the interaction of TAFI with three major components of the extracellular matrix, collagen types I and III, and fibrinogen using surface plasmon resonance. To this end, these matrix components were immobilized on a Biacore sensor chip. Wild−type recombinant TAFI bound to both collagen type I (dissociation constant ~8 nM), collagen type III (~6 nM), and fibrinogen (~135 nM). To determine the regions of TAFI that are involved in these interactions, we studied the binding of several TAFI mutants to the matrix proteins. rTAFI−R302Q, in which the thrombin-cleavage site Arg302 was mutated into a Gln residue, showed similar binding to collagen type I and III compared to wild−type TAFI. TAFI−CPB293−401, a chimera of TAFI and the stable pancreas carboxypeptidase B (CPB), in which the amino acid residues 293−401 of TAFI were replaced by those at corresponding positions of CPB and that was, after activation, 30x more stable than wild−type, showed no detectable binding to either collagen type I or III, or fibrinogen. There was also no detectable binding of CPB to collagen type I and III, or fibrinogen. These data indicate that TAFI binds to matrix proteins via its C-terminal domain. Since collagen and fibrinogen are major determinants of platelet adhesion, we investigated if TAFI influenced platelet adhesion to collagen or fibrinogen. Plastic coverslips were coated with fibrinogen or collagen type III and pre−incubated with buffer or TAFI for 15 minutes at room temperature. Subsequently, a mixture of washed red cells and washed platelets (hematocrit 40%, platelet count 200,000/ul) was perfused over these surfaces at a shear rate of 300s−1 for 5 minutes. Platelet adhesion was quantified morphometrically. Pre−incubation of the collagen surface with TAFI dose−dependently reduced platelet coverage. At a concentration of 10 ug/ml (170 nM) TAFI, which is the approximate plasma concentration, platelet coverage was significantly reduced and complete inhibition was observed at 50 ug/ml. No reduction of platelet deposition was observed on a fibrinogen surface with a concentration of 50 ug/ml TAFI. Pre−incubation of the collagen surface with CPB had no effect. These data imply that TAFI may specifically block the interaction of collagen with the platelet receptors alpha2beta1 or glycoprotein VI, and not the platelet−fibrinogen interaction via glycoprotein IIb−IIIa. In conclusion, TAFI binds specifically and with high affinity to collagens type I and III, and this interaction may be involved in the regulation of platelet adhesion.

Keywords: TAFI, platelets, collagen
Development of a sandwich-type ELISA for the detection of Rat TAFI

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**Introduction:** Activated Thrombin Activatable Fibrinolysis inhibitor (TAFI) attenuates fibrinolysis by removing C-terminal lysines from fibrin. Thereby, their cofactor function in the activation of plasminogen by t-PA is abolished. This antifibrinolytic effect of TAFIa has been demonstrated for both human and rat TAFI in vitro and in vivo. Rat models are frequently used to study thrombolysis. However, immunological tools to quantify rat TAFI in plasma are lacking.

**Objective:** The development of an ELISA for the detection and quantification of rat and murine TAFI in plasma.

**Methods and results:** TAFI knock-out mice were immunized with either recombinant rat TAFI or activated recombinant rat TAFI. The standard hybridoma technique yielded 5 monoclonal antibodies i.e. MA–RT13B2, MA–RT30D8, MA–RT36A3, MA–RT36B2, MA–RT82F12. Twenty-five combinations were pair-wise tested in sandwich-type ELISAs for the detection of rat, murine and human TAFI. Three ELISA combinations i.e. MA–T36A3/MA–T82F12–HRP, MA–RT30D8/MA–RT36A3–HRP and MA–RT82F12/MA–RT36A3–HRP revealed high reactivities towards recombinant rat and murine TAFI and no reactivity towards recombinant human TAFI. Applying plasma to these ELISAs revealed very high reactivity towards rat plasma but no reactivity towards human plasma and TAFI-depleted rat plasma. This high specificity for rat and murine TAFI was further confirmed by Surface plasmon resonance (SPR) analysis. MA–RT82F12 revealed affinity constants of $3.6 \pm 3.4 \times 10^9$ 1/M, $6.6 \pm 2.1 \times 10^8$ 1/M and $9.3 \pm 2.1 \times 10^7$ 1/M for rat, murine and human TAFI, respectively. MA–RT30D8 revealed affinity constants of $6.0 \pm 4.8 \times 10^8$ 1/M, $1.0 \pm 0.1 \times 10^8$ 1/M and $8.0 \pm 1.9 \times 10^7$ 1/M for rat, murine and human TAFI, respectively. MA–RT36A3, the common monoclonal antibody used in these three ELISAs, revealed affinity constants of $2.5 \pm 2.8 \times 10^9$ 1/M, $2.4 \pm 1.4 \times 10^8$ 1/M for rat and murine TAFI and did not bind to human TAFI. All three ELISAs have a lower limit of sensitivity in plasma of 1.25 ng/ml for rat TAFI. Preliminary data revealed TAFI antigen levels of approximately 20 µg/ml in rat plasma.

**Conclusions:** Three sandwich-type ELISAs for the detection and quantification of rat TAFI in plasma were developed. The described ELISAs will be very valuable for the quantification of TAFI in in vitro or in vivo studies on the physiopathological role of TAFI using experimental animal models.

**Keywords:** TAFI, ELISA, rat
A Computer Model of the Fibrinolytic Cascade and its use to Investigate the Inhibition of Fibrinolysis by TAFIa.

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A computer Model, designated “Lyspeed”, has been created to simulate the time courses of all known components of the fibrinolytic cascade from the moment of formation of the fibrin clot until its complete solubilization. The model operates by finding the equilibrium binding distribution of all components at any given point in the process. This distribution is obtained by a process of iteration. From this distribution, rates of the reactions of the cascade are calculated from corresponding rate equations. Rates are then multiplied by a time increment to obtain increments in concentrations. These are then accumulated until the simulated clot is fully dissolved. The number of time intervals typically ranges from 625–1250. The code is written in Visual Basic and executed as a macro in Excel. A full simulation typically takes 2 to 4 seconds. When available, parameter values and rate equations were taken from published work. These included Kd values for the binding of tPA, glu plasminogen and lys plasminogen to intact fibrin, plasmin modified fibrin, and TAFIa treated fibrin. They also included rate equations for the template mechanism exhibited by the activation of plasminogen by tPA with fibrin as a cofactor. The conversion of the glu forms of plasminogen and plasmin to their lysis forms by plasmin, with plasmin–modified fibrin as a cofactor, was assumed to conform to a template mechanism. Inhibition of plasmin and tPA by antiplasmin and PAI–1 included a docking step followed by formation of a covalent enzyme inhibitor complex. For those parameters not available from the literature, best values were found by non-linear regression of experimental data to modeling results with the SIMPLEX procedure. The kinetics of fibrin breakdown by plasmin were inferred by adding fibrinogen at concentrations varying from 2 to 20uM to plasmin and thrombin and monitoring clotting and subsequent lysis over time by turbidity. The kinetics of conversion of the glu forms of plasminogen and plasmin to their lys forms were inferred by fitting modeling results to experiments in which glu plasminogen, lys plasminogen, plasmin–antiplasmin levels and clot turbidity were measured over time in system containing purified components and tPA. The model was utilized to simulate the results of Walker and Bajzar who described a threshold like effect in the suppression of fibrinolysis by TAFIa (JBC 279, 27896–27904, 2004). The model fit the data very well and showed, as found experimentally, that equal increments in the time for a clot to lyse are obtained with each doubling of the TAFIa concentration. They also showed that the size of the time increment is directly proportional to the half life TAFIa for spontaneous decay, as has been shown experimentally. The effect of TAFIa is essentially eliminated if lys plasminogen is included. The simulation also predicts that were TAFIa to be stable or its level sustained by chronic, low level activation by thrombin or the thrombin–thrombo–modulin complex, TAFIa would switch from a transient attenuator of fibrinolysis to a very potent, complete inhibitor. This switch would occur at sub nanomolar to low nanomolar levels of TAFIa.

Keywords: Computer model, fibrinolysis, TAFIa
The Kinetics of Glu- and Lys-Plasminogen Activation on Untreated and TAFIa Treated High Molecular Weight Fibrin Degradation Products

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Plasminogen (Pg) activation to plasmin (Pn) by tissue-type Pg activator (tPA) requires fibrin as a cofactor. Intact fibrin up regulates tPA mediated Glu-Pg activation by approximately 1000-fold compared to no fibrin. Before lysis of the fibrin clot, Pn modifies the fibrin surface resulting in the exposure of carboxyl-terminal lysine and arginine residues. This Pn modified form of fibrin further up regulates Glu-Pg activation by approximately 3-fold over intact fibrin, creating a positive feedback loop for Pn generation. The carboxypeptidase B-like enzyme, activated thrombin activatable fibrinolysis inhibitor (TAFIa), removes these exposed lysine and arginine residues, decreasing the cofactor activity of fibrin to approximately 100-fold less than Pn modified fibrin, thereby attenuating fibrinolysis. While Glu-Pg activation by tPA on plasmin and TAFIa modified fibrin has been characterized, no such analysis has been done with Lys-Pg. Because a fibrin clot is insoluble, soluble high molecular weight fibrin degradation products (HMW–FDPs) were used as a model for plasmin modified fibrin. A fluorescently labeled recombinant Pg mutant, 5IAF–Pg, was used to simplify the kinetic analysis. The active site serine in this Pg derivative has been mutated to a cysteine (S741C), to which 5-iodoacetamidofluorescein has been covalently attached. 5IAF–Pg cleavage does not generate active Pn, thus eliminating the feedback cleavages. In addition, cleavage of this derivative results in an approximate 50% decrease in fluorescence intensity, which can be used to monitor the kinetics of cleavage. Both 5IAF–Glu–Pg and 5IAF–Lys–Pg cleavage by tPA were studied on untreated and TAFIa treated HMW–FDPs. The initial rates of cleavage were fit to a previously derived steady–state template model for tPA mediated Pg activation on fibrin. The catalytic efficiency (kcat/Km) for 5IAF–Glu–Pg cleavage decreased by 94% upon TAFIa treatment of the HMW–FDPs (0.90 microM^-1s^-1 to 0.05 microM^-1s^-1), whereas a 36% decrease in kcat/Km was observed for 5IAF–Lys–Pg (1.38 microM^-1s^-1 to 0.84 microM^-1s^-1). Thus, TAFIa treated HMW–FDPs are an approximately 17-fold better cofactor for 5IAF–Lys–Pg cleavage than 5IAF–Glu–Pg cleavage. This increase in cofactor activity is due to the higher affinity of 5IAF–Lys–Pg for HMW–FDPs than 5IAF–Glu–Pg. TAFIa treatment of the HMW–FDPs resulted in a 62–fold increase in the Kd for 5IAF–Glu–Pg (0.356 ± 0.251 microM to 22.120 ± 472.731 microM). However, TAFIa treatment resulted in only a 2–fold increase in the Kd for 5IAF–Lys–Pg binding to HMW–FDPs (0.356 ± 0.251 microM to 0.721 ± 2.311 microM). Furthermore, a 5–fold increase in the Kd for 5IAF–Glu–Pg binding to HMW–FDPs with bound tPA was observed upon TAFIa treatment (0.079 ± 0.022 microM to 0.387 ± 0.076 microM), but no change was observed for 5IAF–Lys–Pg (0.033 ± 0.017 microM to 0.030 ± 0.029 microM). These data rationalize previous results showing that although TAFIa increases the lysis time of purified clots in the presence of Glu–Pg, it does not do so in the presence of Lys–Pg. This study highlights a potential mechanism for the fibrinolytic cascade to escape the TAFIa mediated prolongation of clot lysis.

Keywords: Plasminogen activation, TAFIa, Lys–plasminogen
A Kinetic Analysis of the Conversion from Glu-plasminogen to Lys-plasminogen by Plasmin in the Presence of Soluble High Molecular Weight Fibrin Degradation Products and the effect of TAFIa on this reaction

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Native or Glu–plasminogen (GPG) can be converted to Lys–plasminogen (LPg) by plasmin (Pn). LPg is 20-fold better as a substrate for tissue–type plasminogen activator (tPA) than GPG and its formation comprises a positive feedback loop that ensures that a mature clot is solubilized in a timely manner in order to avoid excessive fibrin deposition near the site of injury. This conversion of GPG to LPg is enhanced by fibrin (Fn) and to an even greater extent in the presence of plasmin–modified fibrin (Fn’). Although it is known that these fibrin species increase the rate of GPG to LPg conversion, the mechanism and kinetics of this reaction have not yet been fully characterized, the effect of TAFIa on the reaction is unknown. A series of thiol–reactive fluorescent probes were covalently attached to a GPG mutant (S741C) and used to study the kinetics of the conversion from GPG to LPg. The conversion of GPG to LPg is complicated by the fact that Pn cleaves GPG as well as degrades the Fn and Fn’ cofactors. S741C–GPG is an effective tool for analyzing this reaction since cleavage at R560 does not generate active Pn. Experiments were carried out in the presence of soluble high molecular weight fibrin degradation products (HMW–FDPs) that mimic the cofactor activity of Fn’. A time course of the conversion of TMRIA–GPG to TMRIA–LPg was analyzed by acid–urea PAGE and monitored by fluorescence in order to determine mechanistic details of the reaction. Coomassie–blue protein stain shows 2 prominent bands at t = 0. These bands correspond to TMRIA–GPG and FDPs. The identity of TMRIA–GPG is confirmed by exposure to UV light. After 1 minute a third band is evident when exposed to UV light. This band corresponds to TMRIA–LPg. After approximately 30 min the reaction stops. The FDP band had disappeared, likely due to degradation by Pn, and approximately 30% of TMRIA–GPG has been converted to TMRIA–LPg as determined by densitometry. In a purified system containing TMRIA–GPG, the addition of HMW–FDPs was concomitant with a decrease in fluorescence suggesting a binding event between substrate and cofactor. Addition of TAFIa to this purified system causes a subsequent increase in fluorescence back to the level observed for TMRIA–GPG in the absence of HMW–FDPs. This suggests that TAFIa attenuates fibrinolysis by eliminating GPG binding sites on Fn’. TMRIA–GPG also was used to measure by fluorescence the initial rates of cleavage of the 77 residue N–terminal region of GPG by Pn. These data were fit by non–linear regression to a template model. The regression analysis indicated that the template model fit the data well and best fit values for Km, Kd and kcat were returned (360 nanoM, 80 nanoM, 0.40 nanoM, respectively).

Keywords: Glu–plasminogen, Lys–plasminogen, TAFIa
Prognostic value of TAFI level in patients with liver cirrhosis

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TAFI (thrombin activatable fibrinolysis inhibitor) is a potent antifibrinolytic and antinflammatory factor of liver origin and is markedly reduced in liver diseases. We evaluated the prognostic value of TAFI assay in predicting bleeding and mortality in patients with cirrhosis. Sixty–five consecutive patients (53 males, age 29–70 y) of different Child–Pugh class (11 A; 17 B; 37 C) were studied. TAFI antigen was assayed by ELISA (American Diagnostica) and plasma fibrinolytic capacity by evaluating the lysis time of tissue factor–induced plasma clots exposed to 25 ng/ml exogenous t–PA. Assays were performed on samples collected at hospitalization. Both TAFI levels and fibrinolysis time were strongly related to Child–Pugh class (p<0.0001). During 3–year follow–up 32 patients had at least one episode of gastrointestinal bleeding and 25 died because of terminal liver disease. No difference in TAFI level or fibrinolysis time was observed between patients with and without bleeding. Moreover, neither TAFI nor plasma fibrinolytic capacity was associated with bleeding by logistic regression analysis. TAFI levels were significantly lower in non survivors than in survivors (24.8 ± 10.9% vs 38.1 ±10.3%, p=0.0001) whereas fibrinolysis time did not differ between the two groups. In univariate Cox regression analysis, TAFI level (continuous variable), but not plasma fibrinolytic capacity, was strongly associated with survival (b= −0.0671, p<0.0001). In a stepwise multivariate analysis that included age, sex, Child–Pugh class, albumin, bilirubin, factor VII, INR, fibrinogen and platelets, only TAFI and Child–Pugh class were identified as independent risk factors. Using a TAFI cut–off level of 37.3% (derived from ROC curve analysis) TAFI antigen assay showed a highly significant accuracy in assessing patients’ survival (p<0.0001) achieving a sensitivity of 92% (95% CI: 74–99), a specificity of 55% (95% CI: 39–71), and a negative predictive value of 91.7%. By logrank test the risk of fatal outcome in patients with TAFI below 37.3% was 9.2 times higher (95% CI: 2.0–9.9) than in patients with TAFI above this cut–off level (p=0.0002). These data suggest that TAFI is a strong predictor of survival in patients with cirrhosis and may be useful to select candidates for liver transplantation.

Keywords: TAFI, Cirrhosis, survival
The Intrinsic Antifibrinolytic Effect of Carboxypeptidase N is Attenuated by an Inhibitory Component(s) in Plasma

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Thrombosis is a leading cause of morbidity and mortality, causing ischemia, heart attack and stroke. Best practices for treating thrombosis involve rapid recanalization of the impacted vessel, either mechanically (angioplasty) or enzymatically (thrombolytic therapy). Usually, thrombolytic therapy employs exogenous plasminogen activators to catalyze the activation of endogenous plasminogen to plasmin which subsequently proteolyses fibrin, the insoluble proteinaceous component of the pathologic thrombus. tPA, the principal plasminogen activator in vivo, is one of the primary frontline thrombolytic drugs. Plasminogen activation with tPA is enhanced by fibrin cleavage; plasmin cleavage of fibrin generates C–terminal lysines which, by virtue of their affinity for plasminogen and plasmin, respectively, concomitantly increases the rate of tPA–mediated plasminogen activation and decreases the rate of plasmin inhibition. Thus, plasmin generation initiates a positive feedback loop. By removing these C–terminal lysines, basic carboxypeptidases abrogate the feedback loop and thereby down-regulate fibrinolysis. At least two basic carboxypeptidases are found in plasma, TAFIa and CPN. TAFIa, formed up on activation of TAFI, is intrinsically unstable; although extremely efficient, TAFIa–mediated prolongation of clot lysis is limited to about four hours in vitro. CPN is stable but its plasma concentration is too low to affect fibrinolysis. However, CPN can be a potent antifibrinolytic agent; when plasma is supplemented with CPN, clot lysis is not simply delayed as with TAFIa, but is shut down. We recently showed that CPN “activated” by plasmin (CPNa) was much more antifibrinolytic in plasma clot lysis assays than CPN, such that CPNa shut down fibrinolysis at ~1/8th the concentration of CPN. We report here the presence of a component(s) in plasma which inhibits the antifibrinolytic activity of CPN and CPNa, thereby preventing CPN from acting constitutively as an antifibrinolytic in plasma. Several lines of evidence support this hypothesis. First, both CPN and CPNa potently inhibited fibrinolysis at much lower concentrations in clots formed from purified components (3uM fibrinogen, 0.7uM plasminogen, 0.35uM antiplasmin, 0.4nM tPA, 6nM thrombin) as compared to clots formed from dialyzed, 1/3–diluted TAFI–depleted plasma (TdP). The antifibrinolytic effect of CPN and CPNa was about 10–fold greater in the purified system; clots having lysis times of three hours in the absence of carboxypeptidase were prolonged two–fold with 90nM CPN and 15nM CPNa in TdP versus 9nM CPN and 2nM CPNa in the purified system. Second, whereas the concentration dependence of the prolongation of lysis in TdP with CPN and CPNa displayed a long, flat “lag phase” followed by a sharp increase at a threshold concentration, it was steep and essentially linear for both in the purified system. Finally, CPN, but not TAFIa or CPB, was inhibited by plasma from removing C–terminal lysines from partially degraded fibrin in a solid–phase assay and the degree of inhibition was titratable with plasma. Preliminary purification work indicated that the component(s) is proteinaceous. We conclude that plasma contains a component(s) which interacts with CPN and CPNa to prevent CPN–mediated fibrinolytic shutdown. We hypothesize that consumption of the inhibitor(s) in pathologic situations might lead to unregulated CPN–mediated antifibrinolysis, fibrinolytic shutdown and resistance to thrombolytics.

Keywords: carboxypeptidase, fibrinolysis, inhibitor
Characterization of the 48kDa Active Subunit of Carboxypeptidase N and Determination of its Anti-Fibrinolytic Potential

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Thrombus formation, resulting from dysregulation of the coagulation and/or fibrinolytic systems, is the principal cause of heart attack and stroke. Enzymatic treatment of thrombotic events often employs plasminogen activators (PAs) to convert plasminogen to plasmin which degrades fibrin. Degradation of fibrin by plasmin exposes C-terminal lysines which stimulate both plasminogen binding and activation. Activated thrombin–activatable fibrinolysis inhibitor (TAFIa) catalyzes the removal of C-terminal lysine residues present in plasmin–degraded fibrin thereby attenuating up–regulation of plasmin generation. However, TAFIa instability, t? 7 minutes at 37°C, likely limits its antifibrinolytic effect. In contrast, carboxypeptidase N (CPN) is a constitutively active basic carboxypeptidase which circulates in human plasma and appears stable. Further, although CPN and TAFIa share substrate specificity, bradykinin and the anaphylatoxins are substrates for both, an antifibrinolytic role for CPN has yet to be accepted. CPN is a 280kDa dimer of dimers, consisting of two regulatory subunits (83kDa) and two active subunits. The population of active subunits is represented by equal amounts of a 55kDa and a truncated 48kDa form. We previously reported that CPN prolongs fibrinolysis in an in vitro clot lysis model. Proteolysis of CPN by plasmin dissociates CPN into two heterodimers comprising a cleaved regulatory subunit and an active subunit. Compared to CPN, significantly less “activated” CPN enzyme is required to elicit an antifibrinolytic effect. As with TAFIa the active subunit of CPN is reportedly unstable; to infer the role of the regulatory subunit in regulating the antifibrinolytic effect of CPN we determined the stability and antifibrinolytic potential of the purified active subunit. Three molar guanidine HCl followed by gel filtration using Sephacryl 300 was used to dissociate the subunits. As previously described, only the 48kDa form of the active subunit is recovered using this method to purify CPN subunits. As previously shown, intact CPN is stable even when incubated for up to 48 hours at 37°C. The purified active subunit had been shown to lose 25% of its activity after 2 hours at 37°C; however we observed a 4% loss of activity over 2 hours, 27% loss after 24 hours, and 44% loss after 48 hours at 37°C as determined by a chromogenic assay. In a clot lysis assay using purified components (3μM fibrinogen, 0.7μM plasminogen, 0.35μM antiplasmin, 6nM thrombin, and varying tPA concentrations, 50–400pM) or using 1/3 diluted TAFI–depleted plasma (TdP), TAFIa prolonged lysis by 1.9 and 3 hours, respectively. Both CPN and 48kDa subunit in a TdP–clot lysis assay prolonged lysis for greater than 48 hours; however only 15nM 48kDa subunit was required compared to 120nM CPN. In a purified system with 400pM tPA, addition of 50nM CPN or 48kDa subunit prolonged lysis by 2.3 and 7.5 hours, respectively, indicating that the 48kDa subunit is the more potent fibrinolysis inhibitor. In conclusion, the active subunit of CPN is more stable than previously reported and able to prolong fibrinolysis in a comparable manner to native CPN. Dissociation of CPN yielding unregulated active CPN subunits may yield a stable TAFIa analogue capable of long–term thrombus stabilization.

Keywords: Carboxypeptidase N, Fibrinolysis

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Thrombolysis

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**In situ clot formation: An original model for pre-clinical evaluation of stroke therapy**

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Experimental models of focal cerebral ischaemia are a prerequisite to our understanding of the pathophysiology of stroke. Accordingly, a large set of models has been developed in different species to produce cerebral ischaemia. Although global ischemia models are relevant for cardiac arrest, focal stroke models are more immediately relevant for embolism or thrombosis. Some of the models which have been used to promote focal cerebral ischaemia are middle cerebral artery (MCA) occlusion, spontaneous brain infarction in hypertensive animals, embolization and photo-thrombosis. Embolic occlusions can be achieved by injecting either particles like blood clots formed ex vivo, artificial spheres into the carotid artery of animals or by endovascular instillation of thrombin in situ clotting. These models mimic more closely the pathophysiology of human cardio-embolic stroke, but infarct volumes are very variable, and these models are accompanied by a high rate of either spontaneous lysis of injected clots or death. Here, we have developed an original model of in situ clot formation that is compatible with tissue-type plasminogen activator–induced reperfusion. Briefly, male Swiss mice were anaesthetized and ventilated (isoflurane 2% during surgery; with N2O:O2; 3:1). A retro-orbital approach to the middle cerebral artery (MCA) was performed, the dura excised and 1µL of thrombin (0.75 U) was directly injected in the MCA lumen at the level of the first bifurcation through the use of a micro-pipette attached to a stereotaxic device. Physiological and biochemical parameters were monitored and maintained at normal levels (arterial pressure, heart rate, rectal temperature and PaCO₂, PaO₂, pH). Successful MCA occlusion and reperfusion was checked by Doppler sonography. In order to validate the reperfusion model, tissue–type plasminogen activator (tPA), or its vehicle, was intravenously perfused according to the human protocol of thrombolysis (10 % bolus and 90 % during 1 h at 10 mg/kg), 15 minutes after the onset of ischaemia. After 24 h, brains were removed for histological analyses. Although clot formation leads to cortical infarction, tPA induced–reperfusion reduces the severity of the injury. In conclusion, we propose an original model of embolic occlusion which should be useful for pre-clinical evaluation of treatment for human ischaemic stroke.

**Keywords:** cerebral ischemia, plasminogen activator, in vivo model
Anti-platelet aggregation and anti-Helicobacter pylori activity of dipicolinic acid contained in Bacillus subtilis natto

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The fact that the heat resistance of Bacillus subtilis natto is related to the content of dipicolinic acid (DPA) contained within it has drawn attention, along with the anti-blood coagulation activity of Bacillus subtilis natto (Sumi et al., Nippon Nougei Kagakukaishi, 73: 1289, 1999; Ohsugi et al., Food Sci. Technol. Res., 11: 308, 2005). In the current study, substantial differences in the DPA content were found, depending on the Bacillus subtilis natto strain, and the presence of strong anti-bacterial activity (anti-Helicobacter pylori activity) inside Bacillus subtilis natto cells was also found. The strains of Bacillus subtilis natto used in the current tests were the Miyagino, Naruse and Takahashi strains, which are used for the Japanese food natto, a foreign strain (Unnan SL-001) and a strain for medicinal use supplied by Nitto Pharmaceutical Industries, Ltd., and the Meguro Institute. The DPA content was measured using the colorimetric method developed by Janssen and others (Science, 127: 26, 1958), and platelet-rich plasma (PRP) was obtained from a Wistar rat, for measurement of platelet aggregation using an aggregometer (PAT-4A, Mebanix). Minimum inhibitory concentration (MIC) tests (agar plate dilution method) were conducted according to the standard method developed by the Japanese Society of Chemotherapy (Ishii et al., Journal of the Japanese Association for Infectious Diseases, 61: 668, 1987). Measurements of DPA content in each strain of Bacillus subtilis natto showed that DPA content accounted for about 3.6% of the total dry weight of Bacillus subtilis natto in the strain with the highest content. Shaking culture measurements using the same polypeptone-S as the culture medium showed that the strain supplied by Nitto had the highest DPA content at 4.87% of the total dry weight. The addition of glycerol (3.0%) resulted in a value that was 1/220 of this amount, and using nutrient broth as the culture medium resulted in a value that was approximately 1/365. When ADP was used as an aggregation trigger, platelet aggregation patterns were observed, but it was found that adding DPA increases the platelet aggregation inhibition rate on a dose-dependent basis. The CI50 value calculated was 1.8x10^-3M, significantly lower than the case in which aspirin was added (4.0x10^-2M). Natto, a fermented product of Bacillus subtilis natto, was verified to have in itself consider-ably strong anti-Helicobacter Pylori inhibitory activity. In particular, it was confirmed through the MIC tests and the forced administration tests using mice that the ethanol extract of Bacillus subtilis natto (Nitto strain) has strong inhibitory effect against the Helicobacter pylori Sydney strain.

Keywords: Bacillus subtilis natto, DPA, Helicobacter pylori
Regulation of tPA activation and inhibition kinetics by fibrin and fibrinogen

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Background: Tissue plasminogen activator (tPA) is unusual in the coagulation and fibrinolysis cascades in that it is produced as an active single chain enzyme (sctPA) rather than a zymogen. Two chain tPA (tctPA) is produced by plasmin proteolysis at Arg275 but there are conflicting reports in the literature on the behaviour of sc- and tctPA and the significance of this conversion for fibrinolysis. Furthermore, there is little or no work comparing inhibition of sc- and tctPA by the specific inhibitor plasminogen activator inhibitor–1 (PAI–1) under physiological conditions including fibrin(ogen). Aims: To perform a systematic study on the behaviour of sctPA (actually a non–cleavable variant Arg275Glu expressed in insect cells) and tctPA as plasminogen activators and targets for PAI–1 and to determine whether this conversion has any role in the regulation of fibrinolysis.

Methods: Detailed kinetic studies were performed to investigate the behaviour of tPA free in solution and in the presence of template stimulators, fibrinogen and fibrin, using model systems which include native fibrin and partially digested fibrin. Modern approaches of numerical simulation were utilised to cope with the challenges of investigating kinetics of activation and inhibition in the presence of fibrinogen and fibrin.

Results: Enzyme efficiency (kcat/Km) was higher for tctPA than sctPA in solution with chromogenic substrate (enzyme efficiency tctPA/sctPA = zymogenicity, Z=3) and plasminogen (Z=7). Fibrinogen stimulates sctPA kcat/Km (53 fold), more that tctPA (5 fold) bringing Z=1 in the presence of fibrinogen. Z was also close to 1 in the presence of native fibrin and partially cleaved fibrin. sctPA reacts faster with PAI–1 in buffer solution and in the presence of fibrinogen (ratio sc/tc kon=3), and fibrinogen stimulates PAI–1 inhibition of both sc– and tctPA 2 fold. In the presence of fibrin, PAI–1 inhibited 5–15 fold slower than in fibrinogen and there was no difference between sc and tctPA.

Conclusions: Fibrinogen and fibrin modulate the activity of tPA differently in regard to their activation of plasminogen and inhibition by PAI–1. Fibrinogen stimulates both tPA activity against plasminogen and against PAI–1 but fibrin stimulates plasminogen activation (increasing the second order rate constant kcat/Km) while protecting tPA from PAI–1 (decreasing the second order rate constant kon), shifting the balance significantly towards fibrinolysis. Our approaches enable the development of kinetic models of fibrinolysis that address the complex interplay between enzymes, substrates, inhibitors and templates.

Keywords: kinetics, tissue plasminogen activator, PAI–1
Computer models of fibrinolysis

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Background: Kinetic models and simulations exist for the coagulation cascade but not for fibrinolysis. However, such models will be useful in improving our understanding of the regulation of fibrinolysis and improving thrombolytic drugs and therapy. Aims: Utilising our previously published precise methods to study plasminogen activation in fibrin in the presence of chromogenic substrate we initiated efforts to develop a kinetic model and computer simulation to describe plasminogen activation in fibrin clots.

Methods: The initial phase of plasminogen activation takes place in the presence of native fibrin where tPA and plasminogen are loosely associated. As fibrin becomes partially degraded new C-terminal lysine binding sites become available and plasminogen activation is accelerated. Thus modeling begins with a template of native fibrin (without C-terminal lysines) and in order to emulate this situation kinetic experiments were performed using fibrin clots containing carboxypeptidase B. Clots included a range of plasminogen concentrations so we could derive apparent parameters (kcat and Km) for the initial stage of fibrinolysis. Apparent Km and kcat values were derived from data fitting to Michaelis Menten curves or by direct fitting to progress curves using numerical integration. As normal fibrinolysis precedes, C-terminal lysines are generated and plasminogen activation accelerates. Kinetic parameters were determined for partially cleaved fibrin using clots made from fibrinogen pre-treated with plasmin-Sepharose.

Results: In the presence of native fibrin apparent Km and kcat parameters in our experimental system were 417 nM and 0.006 s−1, respectively. In the presence of partially cleaved fibrin the apparent Km drops to 106 nM but the apparent kcat is unchanged. A model was then developed where the real Km of tPA for plasminogen remained constant at 65 micromolar and the changes in apparent Km reflect the increasing local concentration of plasminogen on the fibrin surface. The computer model was developed using Gepasi (a program for modeling metabolic pathways) consisting of 19 reactions and 22 rate constants to simulate the initial phase and subsequent acceleration of plasminogen activation due to the generation of C-terminal lysines. Simulations of tPA-catalysed plasminogen activation by the program resembled raw data and indicated a rate constant for the generation of C-terminal lysines between 1.5 to 5 s−1, which is in line with previously published estimates. The computer model was a little less effective when the activator was Reteplase which we believe reflects the different mechanism of action and heterogeneous nature of plasminogen activation by Reteplase in a fibrin matrix.

Conclusions: Our computer model will be useful in understanding the regulation of fibrinolysis at the molecular level. It can be developed to understand quantitatively how the equilibrium and assembly of components regulates plasminogen activation and fibrin degradation and be used to improve drug design and thrombolytic drug administration.

Keywords: fibrinolysis, regulation, simulation
Inhibition of Various Plasmin Species by Plasma Inhibitors and Its Effect on Clot Lysis

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Active plasmin, a direct fibrinolytic enzyme, is in clinical development as a thrombolytic agent for local, catheter-assisted administration. In contrast to plasminogen activators, plasmin is very effective in lysing long retracted clots, which are naturally deficient in plasminogen. Plasmin is rapidly inactivated by plasma protease inhibitors when it escapes from the clot environment into the circulation. Based upon its unique mechanism of action, plasmin is expected to be a more easily controllable and safer thrombolytic agent than the mechanistic class of plasminogen activators. Several molecular forms of plasmin are known. In addition to natural plasmin which contains 5 kringles (K) attached to a serine protease domain (SP), there are several smaller species, such as Mini-plasmin (K5-SP) and Micro-plasmin (SP only, no kringles). We recently successfully produced a novel, recombinantly-modified, deletion mutant of plasmin, in which the fibrin- and alpha2-antiplasmin-binding kringle 1 is directly attached to the serine protease domain – Delta-plasmin (K1-SP). In this study, we investigated inhibition properties of the above-mentioned plasmin species and effect of their inhibitability on the fibrinolytic activity. Plasmin was a plasma-derived product. Mini-plasmin was obtained by limited elastolysis of plasmin. Micro-plasmin and Delta-plasmin were produced using an E.coli expression system. While catalytic properties of all 4 plasmin species toward a chromogenic substrate S2251 and their inhibition by alpha2–macroglobulin were practically identical, their inhibition by alpha2–antiplasmin was different. Inhibition of Delta–plasmin by alpha2–antiplasmin was as fast as natural plasmin – 10(7) M–1s–1, whereas Micro–plasmin and Mini–plasmin showed expected, ~ 2 order slower inhibition by this fast-acting physiological inhibitor of plasmin. In the next series of experiments, all plasmin species were titrated into plasma in order to determine the plasma inhibitory capacity for each of them. The amount of free plasmin activity in plasma at 4.8 uM of exogenously added plasmin was ranked in the following order from the least to the most: Micro–plasmin < Delta–plasmin < Pm < Mini–plasmin. There were no significant differences in fibrinolytic activity of all species toward washed blood clots (almost no inhibitors). However, when Plasmins were compared in term of their ability to overwhelm plasma inhibitors in the presence of fibrin and initiate clot lysis – they exhibited quite different properties. It required ~3.6 times less amount of Plasmin to initiate clot lysis (1 uM) in comparison with Micro–plasmin (3.6 uM). Delta–plasmin and Mini–plasmin showed intermediate potency, 2 and 2.4 uM, respectively. Thus, in spite of very similar enzymatic properties, the actual fibrinolytic activity of a plasmin molecule depends on several other factors such as its inhibition by plasma inhibitors and by its fibrin binding properties.

Keywords: plasmin, thrombolysis, thrombolytic therapy
Heritability of plasma D-dimer, clot turbidity and clot lysis in the Leeds Family Study

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Aim: The aim of this study was to evaluate the heritability of D-dimer, clot turbidity and clot lysis and to determine the contribution of haemostatic factors and candidate polymorphisms to variance of these measures in 504 subjects from 89 families in the Leeds Family Study.

Methods: D-dimer was measured by ELISA (Biopool). Clot turbidity (lag time and maximum absorbance [max∆abs]) and time to 50% clot lysis (lysis time) were determined by analysing the time course of changes in absorbance of thrombin-induced clot formation and tPA-mediated clot dissolution in microtiter plates read at 340 nm.

Results: The age and sex adjusted heritability of D-dimer was 0.07 (SE 0.09, p=0.13); age and sex accounted for 11.9% of variance, fibrinogen accounted for 6.5% and FXIII 1% of total variance. The age and sex adjusted heritability of lag time was 0.09 (SE 0.07, p=0.09); fibrinogen and FXIII together accounted for 3.3% of total variance. For max∆abs, the age and sex adjusted heritability was 0.22 (SE 0.08, p<0.001); age and sex accounted for 6.7% of variance, fibrinogen accounted for 32%, lag time 4% and tPA 0.5% of total variance. 16.8% of the heritability of max∆abs was unexplained by these variables. In bivariate analysis, there were significant genetic and environmental correlations between fibrinogen and max∆abs (rG=0.491, p=0.029; rE=0.677, p<0.001). The age and sex adjusted heritability of lysis time was 0.21 (SE 0.09, p=0.002); age and sex accounted for 5.4% of variance, and fibrinogen, FXIII, FVII, PAI–1 and lag time accounted for 6%, 2.3%, 3.2%, 9.9% and 5% of total variance, respectively. The FXIII Val34Leu polymorphism accounted for a further 1.3% and the beta fibrinogen –455 G/A polymorphism accounted for 0.5% of variance in lysis time. There was no residual heritability of lysis time after accounting for these variables. Bivariate analyses of clot lysis and haemostatic factors indicated that there was significant genetic correlation between lysis time and fibrinogen (rG=0.667, p=0.002; rE=0.140, p=0.14) and between lysis time and FVII (rG=0.655, p=0.003; rE=0.099, p=0.29). Significant genetic and environmental correlations were found between lysis time and PAI–1 (rG=0.491, p=0.037; rE=0.358, p<0.001).

Conclusions: These data indicate that genetic factors contribute to variance in clot structure and function as assessed by max∆abs and clot lysis time, whereas the determinants of D-dimer and lag time are largely environmental. Common genetic factors contribute to covariance between max∆abs and fibrinogen and between lysis time and fibrinogen, PAI–1 and FVII.

Keywords: D-dimer, clot turbidity, clot lysis
Evidence for an enhanced fibrinolytic capacity in cirrhosis measured with a new global fibrinolysis test in whole blood.

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It is long known that cirrhosis is associated with hyperfibrinolysis, which might contribute to bleeding problems of patients with this disease. However, hyperfibrinolysis has been assessed by using tests (such as euglobulin, plasma or diluted whole blood clot lysis assays), which do not reflect all changes that might occur in the fibrinolytic parameters in blood. A recent study even questioned the presence of a hyperfibrinolytic state in nonbleeding cirrhotic patients, since no evidence of increased fibrinolysis was observed with a new plasma-based clot lysis assay (Gastroenterology 2001;121:131).

Therefore we decided to re-investigate the fibrinolytic state of cirrhotic patients with a recently developed test for global fibrinolytic capacity (GFC) using undiluted whole blood. Non-anticoagulated blood was collected from 30 healthy controls and 75 patients with cirrhosis of varying severity (34 Child-Pugh A, 28 Child-Pugh B and 13 Child-Pugh C). The GFC was determined immediately after collection by clotting the blood, incubating the clots during 3 hours at 37 degrees and then measuring the generated fibrin degradation products. Various haemostatic parameters were determined in plasma samples by using functional activity assays. The median GFC (25th–75th percentile) increased from 1.7 (0.8–3.5) mg/l in the controls to 4.0 (1.6–13.0) mg/l in Child-Pugh A (n.s.), to 11.1 (2.3–31.9) mg/l in Child-Pugh B (P<0.001) and to 22.5 (3.1–89.6) mg/l in Child-Pugh C (P<0.01). Plasma samples of the cirrhotic patients showed significantly decreased levels of antithrombin, plasminogen, alpha-2-antiplasmin and thrombin–activatable fibrinolysis inhibitor (TAFI), significantly increased levels of tissue-type plasminogen activator (tPA) and no significant change in plasminogen activator inhibitor–1 (PAI–1). Spearman correlation tests in the whole group (controls plus patients) showed significant correlations between GFC and antithrombin (r=−0.44), plasminogen (r=−0.38), alpha–2–antiplasmin (r=−0.45), TAFI (r=−0.40), tPA (r=0.77) and PAI–1 (r=−0.51) (all p–values <0.0001). In conclusion, the new GFC test, which includes all components present in blood, indicates that the fibrinolytic capacity is strongly increased in patients with severe cirrhosis. The anti–fibrinolytic effects of decreased plasminogen levels in cirrhosis are apparently overruled by the pro–fibrinolytic effects of decreased alpha–2–antiplasmin and TAFI levels and particularly of increased tPA activity levels.

Keywords: fibrinolysis, global test, liver cirrhosis
Thrombolytic Therapy

Effect of essential oils on the plasma coagulation and fibrinolysis systems

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The effects of the aromatic components of alcoholic beverages and coffee on the fibrinolysis system have been investigated in our previous study (Sumi et al., ISFP Abstract 593, 2004). Essential oils have the recognized therapeutic effect of naturally causing health to be recovered and the psychological effect of inducing a feeling of relaxation. However, there has hitherto been no study investigating the physiological activity of essential oils and their effects on the circulation of blood; in particular, their direct effects on the blood coagulation and fibrinolysis systems. Using many aroma essences as the aromatic components for the tests, the effects of the addition of these essences were investigated in the current study. The essential oils (plant, animal and synthetic essences) were supplied by the R&D Center of Kanebo Cosmetics Inc. (Kanagawa). For the in-vitro tests, 1% Et-OH diluted solution was added, and the standard fibrin plate, the clot lysis time (CLT) and the euglobulin lysis time (ELT) methods were used. For the culture tests, for human cell plasminogen activator (tPA), hepatic cells were cultured by using an E-MEM culture medium in an incubator at 37 °C with 5% CO$_2$. For the platelet aggregation tests, platelet-rich plasma (PRP) obtained from a vein in the tail of a Wistar rat was used, for measurement with an aggregometer (PAT-4A, Mebanix). When 83 types of aromatic components were diluted 1000 times with 1% Et-OH, it was found through the standard fibrin plate, CLT and ELT methods that each of the elder, cashew and grapefruit essences strongly promoted fibrinogenolysis. On the contrary, it was found that the celery, fir, bacca, olive and rosemary essences each had strong inhibitory effects. The effects of the addition of the orange, basil and clove essences to tPA cells were also clearly recognized. In particular, adding 50 microliter of the orange sample to 450 microliter of tPA cell culture solution and incubating for 24 hours at 37 °C with 5% CO$_2$ (first medium) showed that fibrinolytic activity increased to a level eight times greater than that of the control. In the tests concerning effects on platelet aggregation, using adenosine diphosphate (ADP) as the aggregation trigger, strong inhibitory activity was confirmed for basil (77%) and tolu (64%). Platelet--aggregation inhibitory activity was also detected for the essential oils derived from coffee and white peach. Many of the components used in the tests are volatile and are believed to have relatively low molecular weights. With the above--stated effects on blood occurring merely through the smelling of their aromas, it is possible that essential oils could constitute an entirely new category of functional materials.

Keywords: Essential oils, coagulation, fibrinolysis
The streptokinase’s 7 kDa fragment – a possible regulation factor of the fibrinolytic system

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Streptokinase (Sk) is a bacterial origin protein produced by different types of beta-hemolytic streptococcus. Due to the ability to activate the plasminogen – basic pro-enzyme of fibrinolysis system – the streptokinase is widely used in thrombolytic therapy. A general problem of this treatment is a powerful immunogenic activity found after intravenous injection. Besides the wide circulation of the streptococcus infections leads to pre-immunization of the population therefore antibodies to streptokinase can be found at the overwhelming majority of people. Thereof the development of ways of streptokinase immunogenic response decreasing is a priority task of the thrombolytic therapy efficiency. It was shown before that the small fragment formed by streptokinase 1–63 aminoacid residues (MW 7 kDa) did not show a fibrinolytik activity and had not any influencing on this activity of the native streptokinase. Opposite these facts this fragment was capable to stimulate a fibrinolytik activity another both fragments with MW 27 and 36 kDa. So, the using of streptokinase’s functionally active fragments as thrombolytical agents can become the decision of the mentioned above problem. Such opportunity is caused first of all by that some fragments streptokinase’s molecules continue to contain a high fibrinolytic activity and do not include specific antigenic determinants or their immunogenic ability is poorly expressed. Also the using of streptokinase’s fragments for investigation of the fibrinolytic system’s fine biochemical mechanisms will help for discovering of the functional processes of protein–protein interactions in the haemostases system of an animal organism. The purpose of this investigation was found out the influence of the streptokinase’s fragment with molecular weight 7 kDa on the blood plasma fibrinolytic potential expressed by concentration and by activity of the tissue plasminogen activator (tPA) in model system in vivo. We found that streptokinase’s fragments with molecular weight 7 (7 kDa fragment) and 36 (36 kDa fragment) kDa were formed in a result of the limited alpha–chimotrypsin hydrolysis. The 36 kDa fragment was fast degraded to fragments with molecular weight 11 and 17 kDa. The 7 kDa fragment was purified by gel exclusive chromatography on the column with Superdex 75. SDS–PAGE analysis each eluted chromatographic fractions show that one of them included a pure small protein with molecular weight 7 kDa. The Western blot against anti–streptokinase antibodies confirmed the presence 7 kDa fragment in the checked fraction. For investigation a possible activity of 7 kDa fragment on blood plasma fibrinolytical potential the rabbits were intravenously treated by the fragment’s dose equivalent to 20000 IU of streptokinase per 1 kg body (it was approximately 381 ug of fragment for the rabbit by 3 kg weight). The test probes of blood were took per 1, 4 and 24 hours, 4 and 7 day. Collected plasma was controlled on t–PA concentration by enzyme immunoassay analysis and on t–PA activity by colorimetric method with using the specific chromogenic substrate S2251. The both increasing of t–PA concentration and activity in blood plasma after 1 hour 7 kDa fragment injection were shown. The results of this investigation completely confirmed same dates shown before with native streptokinase. Thus, carried out experiments proved a possibility of using streptokinase’s 7 kDa fragment as the indirect thrombolytical agent in the patients treatment.

Keywords: streptokinase, 7 kDa fragment of streptokinas, thrombolytical agent
In vitro effects of two new drugs, Bivalirudin and Desmoteplase, on clot lysis. A comparison with drugs currently used in thrombolysis and revascularisation

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Background New agents such as the bat plasminogen activator (PA) Desmoteplase and the thrombin antagonist Bivalirudin are currently tested for thrombolysis and revascularisation therapies. This study compares these new drugs in their impact on clot lysis with unfractioned heparin and well established thrombolytic agents such as recombinant tissue type PA (rtPA) and Tenecteplase (TNK). Also various combinations of drugs were evaluated.

Methods Whole blood clots (n=10) were incubated and gently agitated in 10 ml PBS at 37°C over 60 min. PBS, which served as control, was compared with Bivalirudin (14 µg/ml) and unfractioned Heparin (0.8 IE/ml). In a second set of experiments the impact of 3 thrombolytic agents on clot lysis was tested: rtPA (5.2 µl/ml) vs. TNK (2 U/ml) vs. Desmoteplase (1.75µg/ml). Furthermore various combinations of these agents were evaluated. From all experiments supernatants were collected and cell culture experiments were performed.

Results Bivalirudin compared to control resulted in a significant increase of clot lysis (10.1% vs. 6.4%; p=0.017) while heparin showed no thrombolytic effect when compared to control. When Desmoteplase was compared to rtPA and TNK, clots were lysed more effectively. When thrombolytics were tested in combination with either Bivalirudin or heparin no additional thrombolytic effect was found.

Summary Our data suggests an additional effect of Bivalirudin on clot lysis when compared to Heparin. The thrombolytic effect of Desmoteplase was more prominent when compared to standard thrombolytic drugs.

Keywords: clot lysis, Bivalirudin, Desmoteplase
Induction of uPA, IL-8 and early growth response-1 by imatinib through activating MAPK in human small cell lung cancer cells

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Purpose: We previously demonstrated that anthracycline antibiotics induced urokinase–type plasminogen activator (uPA) and interleukin-8 (IL-8), a CXC chemokine, in a sublethal dose in human NCI-H69 small cell lung cancer (SCLC) cells where extracellular signal–regulated kinase–1/2 (ERK–1/2) and p38 mitogen activated protein kinase (MAPK) might be involved. NCI-H69 cells generally coexpressed the receptor tyrosine kinase, c-Kit, and the ligand for c-Kit, stem cell factor (SCF). Imatinib is a receptor tyrosine kinase inhibitor against BCR/ABL and c-Kit, and in vitro experiments demonstrated that imatinib inhibited the growth of SCLC cells through inhibition of SCF–induced phosphorylation of c–Kit. We, therefore, investigated the effects of this new agent, imatinib, on the expression of uPA and IL-8 in NCI-H69 cells.

Methods: NCI-H69 cells were cultured in serum-free medium with or without imatinib for 24 hr, then, the cell density was measured by MTT assay. The uPA activities in the cultured medium were measured using a synthetic uPA substrate, S–2444 and a plasminogen–containing fibrin plate (fibrin zymography). The IL–8 antigen levels in the cultured medium were measured using a quantitative enzyme-linked immunosorbent assay kit. RT–PCR and northern blotting were used for detecting the mRNA levels and western blotting was for detecting phosphorylation of MAPKs. Microarray analysis was performed to identify the genes whose expression was influenced by treatment with imatinib.

Results: Imatinib inhibited the cell growth, and surprisingly, treatment with imatinib alone increased the accumulation of uPA and IL–8 in the cultured supernatant of the cells in a dose–dependent manner. Northern blotting and RT–PCR revealed the induction of uPA and IL–8 gene, and the peak induction was observed 9 hr after treatment. Microarray analysis revealed the up–regulation of the gene expression of early growth response–1 (Egr–1) as well as uPA and IL–8 in imatinib–treated cells. The peak induction of Egr–1 gene was observed 2 hr after treatment and the induction rapidly declined. Western blotting demonstrated the phosphorylation of all three MAPKs, such as ERK–1/2, p38 MAPK and stress–activated protein kinase/c–jun N–terminal protein kinase (SAPK/JNK) after treatment with imatinib. U0126, an inhibitor against ERK–1/2, inhibited the IL–8 expression induced by imatinib, whereas either SB202190, an inhibitor against p38 MAPK or JNKI–1, an inhibitor against SAPK/JNK, did not inhibit the expression.

Conclusion: Induction of uPA and IL–8 were observed in imatinib–treated NCI–H69 cells, and the specific inhibitor against ERK–1/2 inhibited the IL–8 expression induced by imatinib, whereas either SB202190, an inhibitor against p38 MAPK or JNKI–1, an inhibitor against SAPK/JNK, did not inhibit the expression. Implication of Egr–1 in IL–8 gene induction was reported and, therefore, we speculate that Egr–1 and ERK–1/2 activation may be involved in the induction of uPA and IL–8 in imatinib–treated NCI–H69 cells.

Keywords: uPA, IL–8, imatinib
Structural basis of uPAR-uPA and uPAR-vitronectin interactions

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Urokinase plasminogen activator (uPA) and its cellular receptor (uPAR) have received extensive study as one of the two primary endogenous systems that mediate plasminogen activation. They are also involved in other general cellular functions and in subsequent diverse pathophysiological processes such as tissue remodeling, arteriosclerosis, tumorigenesis, and tumor metastasis. The uPA binds to uPAR at high affinity (Kd of 0.1–1nM), thus localizing the generation of plasmin from plasminogen onto pericellular regions of a variety of cells. Besides uPA, uPAR is also capable to interact with several other ligands, including vitonectin, uPAR associated protein, various integrins, and G–protein–coupled receptor[1]. The structural origin underlining uPAR’s such capability to recognize multiple ligands is largely unknown. We report here crystal structures of (1) soluble urokinase receptor complexed with the urokinase amino terminal fragment and an anti–receptor antibody at 1.9Å [2]; (2) soluble urokinase–receptor complexed with SomB domain of vitronectin. suPAR is composed of three consecutive domains (D1, D2 and D3) that form a concave shape with a diameter of about 52 Å and a height of 27 Å. At the center of teacup and surrounded by three suPAR domain is a cone shape cavity with wide opening (25 Å) and large depth (14 Å). The amino–terminal fragment (ATF) of urokinase–type plasminogen activator consists of a growth factor domain (GFD) and a kringle domain. GFD domain of uPA ATF inserts into the uPAR cavity. The D1 and D2 domains of uPAR forms hydrogen(644,1017),(657,1023)(644,1018),(657,1024)(644,1019),(657,1025)(644,1020),(657,1026)(644,1021),(657,1027)(644,1022),(657,1028)(644,1023),(657,1029) hydrogen bonds and many hydrophobic interactions with the GFD domain of uPA, thus play an important role in the binding of uPA, which is consistent with previous biochemical studies. However, D3 of uPAR also have some direct interactions with GFD domain of uPA. Kringle domain of uPA sits outside the uPAR pocket, but forms some direct contacts with D1 domain of uPAR. Therefore, three domains of uPAR and two domains of uPA work in cooperation yielding high affinity uPA–uPAR binding. The structure of suPAR in complex with SomB domain of vitronectin shows that the SomB loop 27 that contains double tyrosines (residues 27 and 28) are buried in a cleft form by uPAR 89 and 35 loops from D1 domain. On the other hand, uPAR’s Arg91 inserts into a cleft in SomB domain defined by Asp22, Phe13, Tyr28 and forms hydrogen bonds with Asp22. The structures provide insight into the flexibility of urokinase receptor that enables its interaction with a wide variety of ligands. References: [1] Ploug M. Curr Pharm Des. 2003;9(19):1499–528. [2] Huai Q, Mazar AP, Kuo A, Parry GC, Shaw DE, Callahan J, Li Y, Yuan C, Bian C, Chen L, Furie B, Furie BC, Cines DB, Huang M. Science 2006, 311(5761):656–9.

Keywords: uPAR, Vitronectin, ATF

ID: 86*
Nuclear translocation of urokinase-type plasminogen activator

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Urokinase plasminogen activator (uPA) plays an important role in inflammation, tissue remodeling, angiogenesis and tumor metastasis, in part by regulating extracellular proteolysis, cell adhesion, migration, proliferation, and gene transcription. uPA consists of protease-, kringle- domains and growth factor–like domain (GFD). The latter mediates uPA binding to uPAR/CD87. It has been reported that the isolated kringle domain of uPA can translocate to cell nuclei in vivo and bind specific sequences in DNA in vitro. Therefore, we examined the intracellular trafficking of full-length single chain uPA (scuPA), focusing on the pathway and consequences of nuclear translocation. Using a combination of radioligand, immunocytochemistry, and subcellular fractionation approaches, we observed that full–length scuPA translocates to the nucleus in many cell types where it associates both with DNA/DNA–binding proteins and with nuclear matrix. Nuclear translocation occurs within minutes and does not involve proteolysis or degradation of scuPA. Nuclear translocation is accelerated by uPAR/CD87, the low-density lipoprotein–related receptor and heparan– and chondroitin–sulfate proteoglycans. However, GFD–deficient uPA, which is incapable of uPAR binding, also translocates to the nucleus, albeit less efficiently than full–length scuPA. We found endogenous uPA in the nuclei of human renal tubular epithelial cells and in endothelial cells within the atherosclerotic plaque and neointima in coronary artery. We have shown previously that uPA/uPAR forms a complex with the nucleocytoplasmic shuttle protein nucleolin on the surface of smooth muscle cells, a protein that is involved in regulation of transcription, ribosome biogenesis, and cell proliferation. We found that scuPA binds directly to the C–terminal RGG domain of nucleolin via its kringle domain. Co–localization of exogenous uPA and nucleolin was observed on the cell surface, within the nucleus and in the cytoplasm in HeLa cells and cultured vascular smooth muscle cells. Using lentivirus–mediated RNA interference and mutational analysis, we found that nucleolin is required to transport uPA to the nucleus. uPA binds a cAMP–responsive element (CRE)–binding protein, ATF1, among others, on transcription factor protein microarray. The uPA–ATF1 interaction was confirmed by pull–down and co–immunoprecipitation approaches. Our pilot data show that binding of uPA to ATF1 affects DNA binding as determined by electrophoretic mobility shift assay (EMSA), and affects ATF1–dependent transcriptional activity using luciferase reporter expression. Taken together, these data reveal a novel intracellular pathway by which uPA is rapidly translocated to the nucleus where it may be involved in fundamental aspects of gene regulation and cell proliferation.

Keywords: urokinase, nucleolin, transcription
One-step affinity purification of recombinant uPAR using a synthetic peptide

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Several lines of evidence have pointed to a role of urokinase-type plasminogen activator receptor (uPAR) as a regulator in the processes leading to tumor invasion and metastasis. Consequently, its structure and function has been studied extensively, using recombinantly produced uPAR, purified by either hydroxyapatite or affinity chromatography using the monoclonal anti-uPAR antibody R2 or the cognate ligand urokinase-type plasminogen activator (uPA). Here we present a new method for the affinity purification of recombinant uPAR exploiting a high-affinity synthetic peptide antagonist – AE120. The parent peptide was originally identified in a random phage-display library and subsequently subjected to affinity maturation by combinatorial chemistry. This study compares the affinity purification of a soluble, recombinant uPAR using immobilized monoclonal antibody R2, uPA or AE120. The purity as judged from SDS-PAGE and stability of uPAR purified on the various columns is also investigated. The general availability of peptide synthesis renders the present AE120-based affinity purification of uPAR, exploiting the antagonistic properties of the peptide towards uPAR, more accessible than the traditional protein-based affinity purification strategies. In this way, large amounts of recombinant uPAR can conveniently be purified for further structural and functional studies.

Keywords: uPAR, affinity chromatography, synthetic peptide
Cleavage of uPAR; mechanism and prognostic significance.

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On the cell surface urokinase (uPA) cleaves its receptor, uPAR, thereby inactivating the binding potential of this molecule with regard to both uPA and vitronectin. The cleavage takes place in the linker region between domains I and II of uPAR, after Arg83 and Arg89, liberating domain I, uPAR(I), and leaving the cleaved uPAR(II–III) on the cell surface. uPA cleavage of uPAR is greatly accelerated on the cell surface compared to cleavage of uPAR in solution. This acceleration is dependent on the uPA–uPAR binding. However, uPA does not cleave the uPAR molecule it is bound to but a neighboring molecule. Physiological concentrations of uPA cleave glycolipid anchored uPAR (GPI–uPAR) but not soluble uPAR (suPAR), which lacks the glycolipid anchor. This is due to a difference in the conformation of the linker region between domains I and II and not because of a general difference in proteolytic susceptibility, since GPI–uPAR and suPAR are cleaved with equal efficiency by plasmin. Incubation with an anticatalytic antibody against uPA, clone 5, prevents cleavage on the cell surface, but uPAR(II–III) is found inside the cell even after 48 hours of incubation with anti-uPA clone 5. The total amount of all uPAR forms measured by ELISA in tumor lysates or blood correlates with prognosis in several forms of cancer. However, the amounts of uPAR(II–III) and uPAR(I) may be directly related to the uPA activity and therefore be even stronger prognostic markers. Using combinations of monoclonal anti-uPAR antibodies we have designed 3 time-resolved fluoroimmunoassays for the individual measurements of intact uPAR, intact + uPAR(II–III) and uPAR(I). The amounts of uPAR(II–III) can be calculated. Application of these assays on tumor extracts from 63 patients diagnosed with squamous cell lung carcinoma revealed a stronger prognostic impact of uPAR(I) than of total uPAR, supporting our hypothesis. In plasma from both small cell and non–small cell lung cancer patients we found that the levels of uPAR(I) and uPAR(II–III) were significantly elevated compared to healthy controls, whereas the levels of intact uPAR were similar. In plasma and serum from 32 non–small cell lung cancer patients we also found that the levels of uPAR(I) were significantly associated with survival in a univariate analysis. We will verify this finding in a larger collection of plasma/serum samples from non–small cell lung cancer patients and investigate the prognostic significance and possible diagnostic utility of cleaved uPAR variants in other forms of cancer.

Keywords: cancer, uPAR immunoassays, uPA
Generation and characterization of murine monoclonal antibodies against murine uPAR

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Binding of urokinase plasminogen activator (uPA) to its cellular receptor, uPAR, localizes plasminogen activation to the cell surface. Focal plasminogen activation is involved in both normal and pathological tissue remodelling processes, including cancer invasion. The interaction between uPA and uPAR is therefore a potential target for anti–invasive therapy based on monoclonal antibodies (mAbs). Cancer invasion and metastasis is dependent on the interaction between cancer and stromal cells, which can be studied in genetically induced murine cancer models. To enable in vivo studies of the effect on inhibition of uPA–uPAR binding in genetically induced murine cancer models, we have generated murine mAbs against murine uPAR (muPAR) by immunizing uPAR knock–out mice with recombinant muPAR. We have selected five mAbs (mR1–mR5), which all react with muPAR in ELISA, Western blotting, and Surface Plasmon Resonance analysis. mR1, mR2 and mR4 cross–react with human uPAR in Western blotting. uPAR is a three domain protein, in which the amino–terminal domain I harbors the binding site for uPA, but the entire molecule is required for high affinity interaction. To investigate the epitope location, we subjected muPAR to limited trypsin or pepsin digestion and analyzed the reactivity of the mAbs with the resulting muPAR fragments in Western blotting. mR1, mR3 and mR5 recognized epitopes located in domain I of muPAR, while the epitopes recognized by mR2 and mR4 were located either in domain III, the linker region between domain II and III, or were composed of amino acids in both domain II and III. In cell binding assays, mR1 and mR4 were the strongest inhibitors of uPA binding. mR1 reacts with domain I and all previously characterized mAbs reacting with epitopes on domain I in human uPAR affect the uPA–uPAR interaction. mR4 recognizes muPAR(I+II–III) as does mR2, demonstrating that also this part of the molecule is important for the uPA binding. Interestingly, mR3 showed no sign of inhibition of the muPA–muPAR interaction even though it reacts with domain I. In vivo administration of mR1 to mice deficient in tissue plasminogen activator (tPA) resulted in accumulation of fibrin deposits in the liver sinusoids, similar to the phenotype previously observed in uPAR/tPA double–deficient mice (Bugge et al., 1996). The mAbs generated here will be further characterized in order to select those that will be optimal for therapeutic studies.

Keywords: Cancer invasion, Plasminogen activation, Therapeutic target
Inhibition of mouse uPA activity by mouse monoclonal antibodies


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Deficiency in urokinase plasminogen activator (uPA) in mice is associated with >7-fold reduction of lung metastasis in the MMTV-PymT transgenic breast cancer model as well as reduced dissemination to brachial lymph nodes. In contrast, tumor incidence, growth rate, and final primary tumor burden are not significantly affected by uPA deficiency in this model (Almholt et al., 2005). The potential of monoclonal antibodies (mAbs) as anti-cancer therapeutics has lately been demonstrated for a number of antigens. As the clinical relevant mAbs are directed against human antigens, the initial in vivo studies in mice have been limited to xenotransplanted tumors, which generally are poor models for human cancers, since they do not reflect the interaction between tumor and stromal cells. In this respect genetically induced murine cancer models are superior. To enable in vivo therapy experiments using mAbs in murine cancer models, we have utilized the fact that mice deficient in a particular protein, such as murine uPA (muPA), produce specific murine antibodies against muPA upon immunization with the recombinant muPA protein. Thus, the generated murine anti–muPA mAbs can be tested for their effect on tumor growth and metastasis in murine cancer models. We have selected 5 mAbs (mU1–mU5) reacting with muPA in ELISA, Surface Plasmon Resonance (SPR) analysis, and Western blotting, while no cross-reactivity with human uPA was observed. The reactivity of the mAbs was analyzed using the recombinant amino-terminal fragment of muPA (mATF), revealing that all but mU1 recognized epitopes in mATF. Thus, the epitope of mU1 is located in the B-chain, encompassing the catalytic site of muPA. SPR analyses demonstrated that mU2 was unable to bind receptor (muPAR)–bound muPA and prevented binding of muPAR to muPA, indicating overlapping binding sites of muPAR and muU2 on the muPA growth factor domain. The 3 residual mATF-reacting mAbs bound muPA independently of muPAR–binding. Using an enzyme kinetic assay measuring muPA–dependent plasminogen activation, mU1–mU4 were found to inhibit the catalytically activity of muPA to various extend, with mU1 having the most pronounced effect. In contrast, mU5 stimulated the reaction. Notably, in vitro cell binding experiments using 125I–mATF illustrated an efficient mU2–induced interference of the muPA interaction with muPAR on the cell surface. Application of mU1 in an anthrax–toxin assay dependent on muPA– activity yielded nearly complete cell rescue and importantly, treatment of tissue–type plasminogen (tPA) deficient mice with mU1 resulted in significantly delayed wound healing mimicking the phenotype observed in uPA/tPA double–deficient mice. Hence, mU1 will now be the first of these anti–muPA mAbs to be tested for its effect on tumor growth and metastasis in murine cancer models.

Keywords: cancer, plasminogen activation, therapeutic targeting
The Role of uPAR and Alpha 3-Beta 1 Integrin in the Modulation of Integrin Function

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Human oral squamous cell carcinoma (SCC) represents approximately 90% of all oral cancers and in the United States, nearly 29,000 people are diagnosed with the disease each year. Elevated expression of the urinary plasminogen activator (uPA) and its receptor uPAR are hallmarks of the disease phenotype. Present on the mammalian cell surface, uPAR has been implicated in cell adhesion, migration, and proliferation. uPAR is connected to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor. Although uPAR lacks transmembrane and intracellular domains, the receptor overcomes its structural limitations by acting as a lateral ligand to modulate intracellular signaling in response to external stimuli. Previous studies have identified integrins, including the alpha 3-beta 1 heterodimer, as proteins that physically associate with uPAR to regulate migration, adhesion, and cell-cell contact in human epithelial cells. While the binding of uPAR to alpha 3-beta 1 is well characterized, less is known about the molecular mechanisms employed by uPAR during signal transduction in oral cancer metastasis. Evidence suggests that uPAR may exist as part of a multiprotein complex containing the 22 kDa scaffold and integral membrane protein caveolin. As a component of lipid rafts, localized membrane microdomains enriched in signaling proteins, caveolin has been shown to co-immunoprecipitate with beta 1 integrin in oral cancer and function as a tumor suppressor by maintaining Src in an inactive conformation. Oligomerization of caveolin releases Src to promote proliferation and cancer progression through activation of the mitogen-activated protein kinase (MAPK) signaling pathway. In this study, sucrose density centrifugation has been utilized to demonstrate that uPAR, alpha 3 and beta 1 integrins, Src, and caveolin are present in the same membrane raft fraction under basal conditions. uPAR interacts with alpha 3 integrin directly, as suggested by immunoprecipitation (IP) and additional IP experiments are underway to further evaluate protein:protein interactions in raft fractions. If a complex forms consisting of uPAR and integrins, then the distance between the interacting partners is predicted to be less than 10 nm. To test this hypothesis, fluorescence cytometric energy transfer (FCET) is currently being employed using antibodies conjugated to donor and acceptor fluorophores to evaluate complex formation on live cells. Previous research from the laboratory has demonstrated that the uPAR/alpha 3-beta 1 integrin interaction modifies integrin signaling. Oral SCC cells that overexpress uPAR or have stably downregulated uPAR levels (using siRNA) have been generated and are currently under evaluation in a panel of assays to assess migration, adhesion, and cell spreading. Because integrins interact with the cytoskeleton and surrounding extracellular matrix (ECM), the modulation of uPAR levels is predicted to impact the ability of oral epithelial cells to adhere to or traverse ECM barriers. This is supported by results obtained from a Matrigel invasion assay, suggesting that the ability of oral SCC cells to invade the ECM is contingent upon uPAR expression. A better understanding of the functional ramifications resulting from uPAR binding to its signaling partners will shed new insight into oral cancer metastasis and aid in the development of new therapeutic strategies.

Keywords: uPAR, integrin, OSCC
Macrophage-Expressed Urokinase-type Plasminogen Activator is an Important Modifier of Atherosclerosis

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Purpose of study: Urokinase-type plasminogen activator (uPA) is expressed in human atherosclerotic lesions, primarily by macrophages; however, whether macrophage–expressed uPA alters the severity of atherosclerosis is unclear. We previously showed that apolipoprotein E null mice (Apoe−/−) with a macrophage–targeted uPA transgene (SR–uPA+/0) have accelerated atherosclerosis, severe proximal coronary stenoses, and early mortality (all dead by 30 weeks). However, it was not clear in this model whether uPA–overexpressing macrophages were the critical factor in accelerating atherosclerosis. In addition, our finding that increased uPA expression was pro–atherogenic contrasted with an earlier report in which complete loss of the uPA gene (Plau) had no effect on the extent of atherosclerosis in 37–week–old Apoe−/− mice (Carmeliet et al. Nat. Genet. 1997). The purpose of this study was to more precisely test the role of increased and decreased macrophage uPA expression on the severity of murine atherosclerosis.

Hypothesis: We hypothesized that macrophage–expressed uPA is an important modifier of atherosclerosis, and therefore that transplantation of uPA–overexpressing macrophages would accelerate atherosclerosis and transplantation of uPA–deficient macrophages would retard atherosclerosis.

Methods: To increase macrophage uPA expression, Plau+/+ Apoe−/− mice were lethally irradiated and transplanted with bone marrow (BM) from SR–uPA+/0 Apoe−/− mice. To ablate macrophage uPA expression, Plau+/+ Apoe−/− mice were lethally irradiated and transplanted with BM from Plau−/− Apoe−/− mice. In both studies, control Plau+/+ Apoe−/− mice were lethally irradiated and transplanted with BM from Plau+/+ Apoe−/− mice. All mice were in the C57BL6/J background. Study 1: Donor Recipient Experimental SR–uPA+/0Apoe−/− to Plau+/+ Apoe−/− Control Plau+/+ Apoe−/− Study 2: Experimental Plau−/− Apoe−/− to Plau+/+ Apoe−/− Control Plau+/+ Apoe−/− to Plau+/+ Apoe−/− Male BM was transplanted into 8–week–old female recipients (n=10–14). Reconstitution was confirmed by Y chromosome–specific PCR of peripheral blood DNA.

Recipients were fed a Western diet (0.15% cholesterol, 21% fat, no cholate) at 12 wks of age and euthanized at 22 wks (Study 1) or 37 wks (Study 2). We measured plasma lipids and FPLC profiles. Peripheral blood monocytes were measured in Study 2. Atherosclerosis was quantified by en–face analysis of pinned whole aortae in both studies and cross–sectional analysis of aortic roots in Study 1.

Results: Plasma lipids, FPLC profiles, and peripheral blood monocyte counts were not affected by variations in macrophage uPA expression. Recipients of SR–uPA+/0 Apoe−/− BM had 2–3–fold more atherosclerosis than controls (24 ± 16 versus 9.9 ± 3.4% aortic plaque coverage; P < 0.001 and 0.76 ± 0.30 versus 0.28 ± 0.17 mm2 aortic root lesion area; P < 0.001). Recipients of Plau−/− Apoe−/− BM had 35% less aortic atherosclerosis than controls (21 ± 10 versus 31 ± 13% aortic plaque coverage; P = 0.034; aortic root analysis is pending).

Conclusion: Macrophage uPA expression—over a broad range—modifies the progression of atherosclerosis in Apoe−/− mice. Therefore, inhibition of uPA expression in macrophages or antagonism of uPA activity in the artery wall are potential strategies for prevention and treatment of atherosclerosis.

Keywords: urokinase, atherosclerosis, macrophages
Initial Angiogenic Cell Migration Depends on CD87-D3 Mediated Integrin Internalization


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Angiogenesis, the formation of new blood vessels after injury or in neoplastic disease, is initiated by growth factors stimulating migration of endothelial cells. Cell migration requires formation of cell-matrix contacts by integrins at the leading edge, disengagement of these receptors at the trailing edge and thereafter their redistribution. It is largely unknown though, how integrins are internalized during this process. We here show how beta-1 integrins become internalized upon stimulation of endothelial cells by the angiogenic vascular endothelial growth factor (VEGF). We have shown previously that VEGF, by interacting with its receptor-2, induces activation of CD87 bound pro-urokinase (pro-uPA) for local proteolytic matrix degradation. Once the activity of uPA becomes blocked by the matrix residing specific plasminogen activator inhibitor-1 (PAI-1), the trimolecular complex CD87/uPA/PAI-1 formed becomes internalized via a member of the LDL receptor family. We now show that, at the same time, VEGF stimulation leads to internalization of integrins as well. By interacting with domain 3 of CD87, beta-1 integrins are co-internalized into the endosomal compartment, but in contrast to internalized uPA/PAI-1, they are not degraded by the proteasome. The internalization of this complex can be inhibited either by the use of RAP, an inhibitor of LDL–R family ligand interaction, or by depletion of uPAR from the cell surface by cleaving the GPI-anchor of uPAR by PI–PLC, or by the use of peptides derived from domain–3 of CD87 that inhibit either CD87 – beta-1–integrin or CD87 – LDL–R family member interaction. Using either of these measures, endothelial cell migration in vitro and angiogenesis in a mouse Matrigel® model in vivo is severely impaired. These data imply that CD87 contributes to VEGF induced migration by aiding integrin internalization. Targeting CD87 beta-1 integrin interaction might be a novel approach for an anti-angiogenic therapy.

Keywords: uPAR, integrin, angiogenesis
Urokinase (uPA) induces cell survival by up-regulating inhibitor of apoptosis proteins (IAPs)

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The urokinase system has originally been implicated to assist the angiogenic process by its proteolytic properties. But it is now becoming increasingly evident that uPA elicits many pro-angiogenic responses like differentiation, proliferation and cell migration in a non-proteolytic fashion. In this study we demonstrate that uPA protects against apoptosis by inducing the NFκB pathway. Thereby, uPA-induced activation of NFκB leads to a transcriptional upregulation of inhibitor of apoptosis proteins (IAPs), among them most prominently the X-linked inhibitor of apoptosis protein (XIAP). Blocking NFκB using the specific NFκB inhibitor BAY 11–7082 or via adenoviral mediated overexpression of its inhibitor, IkB, inhibits uPA-induced XIAP expression as well as uPA-induced cell survival. When we compared the main angiogenic growth factor VEGF and uPA, we found that only uPA elicits anti-apoptosis independently of the PI3kinase pathway and, furthermore, is able to rescue apoptosis induced by PI3-kinase inhibition. Thereby, uPA induces a CDC42 dependent phosphorylation of its downstream effector p21-activated kinase 1 (PAK1), which leads to IkappaB kinase alpha (IKKa) phosphorylation, a prerequisite for NFκB activation. The uPA induced NFκB activation is, however, only seen in matrix adherent cells and was absent on cells seeded on polylysine, indicating the involvement of integrin-mediated signalling necessary for uPA dependent NFκB activation and upregulation of IAPs. From these data we conclude that uPA activation, which is a main player in endothelial cell migration and invasion, provides an additional, PI3-kinase independent cell survival mechanism.

Keywords: uPA, NFκB, anti-apoptosis
The density enhanced phosphatase 1 (DEP-1) down-modulates urokinase receptor (uPAR) surface expression in confluent endothelial cells

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We have shown previously that VEGF165 the pivotal endothelial cell growth factor initiates endothelial cell migration by VEGFR-2 and PI-3 kinase dependent pro-uPA activation. Activation takes place bound to uPAR and involves an integrin-dependent MT1-MMP and MMP2 activation (Prager et al, Blood 2004); active uPA in turn is inactivated by its inhibitor PAI-1 and the uPAR–uPA–PAI-1 complex is internalized via a LDLR member. This process, however, is limiting for endothelial cell migration in vitro and angiogenesis in vivo in a Matrigel® plaque model in mice (Prager et al Circ. Res. 2004). The amount of surface uPAR is therefore important for the initial response of endothelial cells towards angiogenic stimulation. Consistently, we have found that confluent cultures of endothelial cells express less (~60%) uPAR on the surface as compared to sub-confluent cultures. Human density enhanced phosphatase-1 (DEP-1) a member of receptor-like tyrosine phosphatases family consists of an extracellular domain including eight fibronectin III domains, a transmembrane segment and an intracellular tail with a single protein tyrosine phosphatase domain. As indicated by its name, the expression of human DEP-1 increases in dense cell cultures, providing an inhibitory effect on cell growth. We could show that also in endothelial cells DEP-1 expression is dependent on cell density as revealed by QT-PCR, flow cytometry and fluorescence microscopy. DEP-1 is also crucial for the developing vasculature, since mice with disrupted DEP-1 gene suffer from early embryonic lethality at midgestation due to impaired vascularisation. This prompted us to analyze a possible relation between DEP-1 and the uPAR system. We therefore determined the effect of overexpression of DEP-1 on uPAR expression and surface distribution. We generated DEP-1 overexpressing plasmids encoding full length DEP-1, full length DEP-1 with a C >>S mutation in the catalytic region, as well as constructs expressing only the extracellular or the intracellular domains of DEP-1. Upon transfection of HUVECs with these different plasmids, full length DEP-1 caused in subconfluent cells 30% decrease in uPAR expression on the surface of HUVECs when compared to the phosphatase mutated or phosphatase domain deleted forms. In addition, this effect of DEP-1 overexpression on uPAR expression was restricted to the active phosphatase linked to the membrane via its extracellular domain indicating that general increase in phosphatase activity is not sufficient to induce the decrease in uPAR expression. To further exploit this phenomenon, we studied the effects of ERK1 overexpression or of the MAP-kinase inhibitor PD 098059. Neither was the effect of DEP-1 overexpression reversed nor mimicked by these two measures. This indicates that full length membrane bound DEP-1 is responsible for the decrease in uPAR expression. As final prove we have generated RERT mDEP−/− knock-out mice that will be used to determine uPAR expression in response to cell density. In conclusion these data indicate that uPAR surface expression is regulated by DEP-1 dependent on phosphatase activity and membrane anchor. Such mechanism should allow fine tuning of the response of endothelial cells towards an angiogenic stimulus.
Mechanisms of Urokinase-Mediated Vasoconstriction

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Purpose of Study: Urokinase plasminogen activator (uPA) is expressed at increased levels in atherosclerotic human arteries; however, the biological roles that uPA plays in the artery wall are incompletely understood. We showed previously that overexpression of uPA in carotid arteries of cholesterol-fed rabbits causes arterial constriction (Falkenberg et al PNAS 2002;99:10665-70). However, it was unclear whether constriction was an acute vasomotor effect or persistent inward remodeling. It was also unclear which domain of uPA was responsible for causing arterial constriction and whether hyperlipidemia was required for uPA-mediated arterial constriction. Previous studies have associated vasoconstrictor activity with the kringle domain of two-chain uPA (Nassar et al. JBC 2002;277:40499-504) not with the protease domain. Hypothesis: uPA-induced arterial constriction is a reversible vasomotor process that requires proteolytic activity and is independent of hyperlipidemia.

Methods: We constructed rabbit uPA mutants that lack either the N-terminal receptor-binding domain (uPAdel) or proteolytic activity (uPASer-Ala). We constructed first-generation adenoviral (Ad) vectors that express wt uPA or the uPA mutants. In vitro Ad-expressed uPA was characterized by western blot, plasminogen activation (PA) assay, and cell–binding studies (n=5–6). Arteries of chow-fed rabbits were transduced with Ad expressing wt uPA, the mutant uPAs, or AdNull (no transgene). Arterial uPA expression was measured by western blot and uPA activity assay performed on explant culture medium (n=4–6). Vasomotor activity was detected by application of papaverine (n=14). Artery size was measured by perfusion-fixation and planimetry (n=8–9). Data are presented as mean ± S.D.

Results: Secreted PA activity was similar for cells transduced with wt uPA and uPAdel (3.4 ± 1.0 and 2.5 ± 0.2 IU/ml/mg). uPASer–Ala had no detectable PA activity (<0.01% of wt). Cells treated with uPAdel had less cell–bound uPA protein and uPA activity than cells treated with wt uPA. In vivo, arteries transduced with wt or mutant uPA expressed similar amounts of uPA protein. Arteries transduced with wt uPA and uPAdel had increased uPA activity; AduPASer–Ala arteries did not. AdwtuPA arteries were constricted compared to AdNull arteries [internal elastic lamina (IEL) length = 3.1 ± 0.4 vs. 3.9 ± 0.3 mm; P<0.001]. Papaverine completely reversed the constriction (IEL = 3.9 ± 0.7 vs. 4.0 ± 0.4 mm for AdNull arteries; P=0.4). In an experiment specifically testing the mutants, uPASer–Ala did not cause constriction (IEL = 4.3 ± 0.4 vs. 4.2 ± 0.7 mm for AdNull; P=0.9). AduPAdel arteries were as constricted as AduPA arteries (IEL = 3.6 ± 0.37 versus 3.3 ± 0.47 mm for AduPA arteries; P = 0.2) Conclusions: uPA–mediated constriction is a reversible vasomotor process that requires proteolytic activity and is independent of hyperlipidemia. We speculate that uPA proteolytic activity may contribute to lumen loss in atherosclerotic arteries. Further identification of the downstream mediators of uPA–induced vasoconstriction may reveal novel pathways that regulate vascular tone and affect luminal area.

Keywords: Artery, Urokinase, Vasoconstriction
Macrophage-Expressed Urokinase-Type Plasminogen Activator Accelerates Atherosclerosis Progression in Two Mouse Models

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Purpose of study: Urokinase-type plasminogen activator (uPA) is expressed in human atherosclerotic lesions, primarily by macrophages. We previously showed that apolipoprotein E null (Apoe−/−) mice with a macrophage–targeted uPA transgene (SR−uPA+/−/0 mice) exhibit accelerated atherosclerosis, medial wall destruction, and severe coronary stenoses at 15 weeks of age, and die prematurely. In the Apoe−/− background, the SR−uPA transgene increases atherosclerosis both on pinned aortas and aortic roots and does not affect plaque morphology. To determine whether uPA modifies atherosclerosis in another mouse model we bred the SR−uPA+/−/0 transgene into mice that are deficient in the LDL receptor (Ldlr−/−).

Hypothesis: We hypothesized that the SR−uPA transgene would also accelerate atherosclerosis in 15-week-old Ldlr−/− mice.

Methods: SR−uPA+/−/0 Ldlr−/− and non–transgenic Ldlr−/− mice were fed a Western diet (0.15% cholesterol, 21% fat) starting at five weeks of age and were euthanized at 15 weeks. Atherosclerosis was quantified by en face analysis of pinned aortas and H&E staining of aortic root sections. Aortic root plaque morphology was analyzed by ORO staining for lipids, by MOMA-2 immunohistochemistry for macrophages, and by MOVAT staining to detect medial wall destruction. uPA expression was measured in macrophage–conditioned media. Plasma lipids and peripheral blood monocytes were quantified using standard methods. Mice of both groups were monitored for lifespan.

Results: SR−uPA+/−/0 Ldlr−/− mice had increased aortic root atherosclerosis compared to non–transgenic littermate controls (0.44 vs 0.27 mm²; P=0.034) but no change in % plaque area measured on pinned aortas (4.7 vs 4.1%; P=0.32). Interestingly, aortic root plaques in SR−uPA+/−/0 Ldlr−/− mice exhibited a significantly greater percentage ORO-positive area (61 vs 51%; P=0.019), a significantly smaller percentage MOMA-2 positive area (21% vs 32%; P=0.006), and no significant medial wall destruction. Coronary stenosis was rarely found in either SR−uPA+/−/0 Ldlr−/− or nontransgenic mice at 15 weeks. Furthermore, SR−uPA+/−/0 Ldlr−/− mice died prematurely beginning at 23 weeks of age, rather than at 15 weeks, as we had found in Apoe−/− mice. No significant difference was found in the level of uPA activity in macrophage–conditioned media in Ldlr−/− vs Apoe−/− mice. Plasma lipids and peripheral monocyte counts were unaffected by the SR−uPA transgene in the Ldlr−/− background.

Conclusion: Macrophage–expressed uPA accelerates atherosclerosis in Ldlr−/− as well as Apoe−/− mice. The smaller lesion areas in Ldlr−/− mice—especially on pinned aortas—and the lack of coronary stenoses or medial destruction in the Ldlr−/− mice as well as the later onset of premature death in the Ldlr−/− background all suggest an earlier stage of atherosclerosis at 15 weeks in SR−uPA+/−/0 Ldlr−/− vs SR−uPA+/−/0 Apoe−/− mice. We speculate that macrophage overexpression of uPA has a greater effect on progression than on initiation of atherosclerosis. Moreover, the increased percent lipid area in lesions of SR−uPA+/−/0 Ldlr−/− mice suggests that uPA accelerates lesion progression in Ldlr−/− mice by increasing artery wall lipid retention. We are currently testing a prediction that uPA–mediated acceleration of atherosclerosis will be more evident in older Ldlr−/− mice.

Keywords: atherosclerosis, macrophages, urokinase
Wound Healing

**ID:** 275*

**The plasminogen activation system and skeletal muscle regeneration**

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Skeletal muscle regeneration involves extensive tissue remodeling. The plasminogen activation (PA) system is a widely used mechanism for the generation of proteolytic activity in the extracellular matrix, contributing to a variety of tissue remodeling processes. We have observed that the PA system components play differential roles in muscle regeneration after injury: uPA and plasmin activities are necessary for this process, whereas that of tPA is dispensable, indicating that no redundancy exists between both PAs in muscle; in contrast, PAI–1 deficiency improves the muscle regeneration process. Moreover, we have demonstrated that uPA deficiency exacerbates muscular dystrophy in mdx mice (the most widely used animal model for Duchenne Muscular Dystrophy –DMD) as indicated by morphological, biochemical and functional parameters. Mdx/uPA–/- mice exhibited increased muscle degeneration and fibrin deposition as well as reduced extent of regeneration; interestingly, pharmacological depletion of fibrinogen rescued significantly the exacerbated dystrophic phenotype of mdx/uPA–/- muscle. Altogether, these findings indicate that uPA–mediated fibrinolysis is necessary for muscle regeneration mdx mice.

**Keywords:** plasminogen activation, muscle regeneration, fibrin

* selected for oral presentation
† not represented at ISFP Congress
Bone Marrow-derived PAI-1 Influences the Development of Obesity

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The visceral fat mass is an important correlate and producer of plasminogen activator inhibitor–1 (PAI–1). While adipocytes are capable of synthesizing PAI–1, the bulk of evidence at present indicates that stromal cells in visceral fat are the primary source of PAI–1. We hypothesized that PAI–1 derived from macrophages contributes to the development of diet–induced obesity. To test this hypothesis, 6–to 8–week–old male C57BL/6 wild–type (WT) and PAI–1–deficient mice on a C57BL/6 background (KO) were transplanted with either PAI–1+/+, PAI–1+/- or PAI–1–/– bone marrow (n=5–10). The transplanted animals were fed a high fat diet (HFD) for 24 weeks. Animals were weighed and venous blood samples were collected at 2 week intervals. At the conclusion of the study, the mice were sacrificed and the abdominal visceral fat was collected. WT hosts had significantly higher PAI–1 mRNA levels in their epididymal fat pad than the different transplantation combinations in the KO hosts (p<0.0001). In WT mice receiving PAI–1+/+ bone marrow PAI–1 mRNA levels were 5.8– and 2.8–fold higher in comparison with those receiving PAI–1+/- and PAI–1–/– marrow respectively (p<0.05). Results from the transplanted KO hosts confirmed the contribution of bone marrow–derived cells. KO hosts receiving PAI–1–/– bone marrow gained less weight than all other host/donor combinations (p<0.05), and retained a reduced body composition as well (p<0.05). The epididymal fat pad weight was 2–fold lower in the KO hosts receiving PAI–1–/– bone marrow in comparison with the other groups (0.8±0.1 g vs 1.6 to 1.9 g; p<0.05). Remarkably, even though the WT/PAI–1–/– animals were not protected from overall weight gain, they had less visceral fat (1.2±0.1 g). The ratio stromal cells/adipocytes was significantly different between the host/donor groups (p<0.01) with least stromal cells for the KO and WT hosts receiving PAI–1–/– marrow. Also adipocyte size was reduced in these two groups (p<0.5). In order to quantify the number of macrophages present in the epididymal fat pad, we analyzed the relative expression of CD68 mRNA using RT–QPCR. The number of macrophages in the fat pad differed between the groups (p<0.05), with KO and WT hosts receiving PAI–1–/– bone marrow exhibiting 2.9– and 7–fold lower CD68 mRNA levels than WT hosts receiving PAI–1+/+ bone marrow (p<0.001). When comparing the different KO host/donor combinations, a “bone–marrow derived PAI–1” concentration effect on CD68 mRNA levels appeared. In conclusion, the present study shows that bone marrow–derived PAI–1 influences the development of obesity, an observation that mechanistically includes an effect on macrophage accumulation in visceral adipose tissue. This study demonstrates that bone marrow constituents play a role in coordinating the accumulation of visceral fat and suggests that cellular and humoral factors originating in the bone marrow influence the development of obesity. As such, PAI–1 can be defined as a potential therapeutic target, not only to prevent obesity development, but also metabolic disturbances associated with it.

Keywords: PAI–1, obesity, inflammation
Effect of ochratoxin A on tissue factor and PAI-2 production by human blood mononuclear cells

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The mycotoxin ochratoxin A (OTA) is a ubiquitous contaminant of human and animal food products. Apart from its known nephrotoxicity and carcinogenicity, OTA has been shown to variably affect several functions of mononuclear leukocytes. We have studied the effect of OTA on tissue factor (TF) and plasminogen activator inhibitor–2 (PAI–2) production by peripheral blood mononuclear cells (MNC) stimulated with endotoxin (1 microg/ml, 3 h and 18 h at 37°C for TF and PAI–2, respectively). TF was measured by functional (one-stage clotting time) and immunological (ELISA) assays, and by RT–PCR whereas PAI–2 was assessed by ELISA in conditioned media. OTA caused a dose–dependent reduction in TF activity and antigen (with more than 90% inhibition at the concentration of 1 microg/ml) and also reduced PAI–2 release (80% inhibition at 1 microg/ml). Inhibition of TF expression was also observed at mRNA level. The inhibitory effect disappeared if OTA was added to MNC suspensions 20–60 min after endotoxin. Moreover, OTA was much less efficient in reducing TF expression when MNC were suspended in medium containing 40 mg/ml human albumin. TF production was also impaired by OTA (1 microg/ml) when MNC were stimulated with 10–9 M PMA (99% inhibition), 10 ng/ml IL–1beta (84%) or 100 ng/ml TNF–alpha (55%). Finally, we determined the effect of OTA on endotoxin–induced cytokine release by MNC and found that OTA inhibited IL–6, but not IL–8 or TNF–alpha production, thus ruling out an unspecific effect of the mycotoxin on protein synthesis. Because of the important role of blood clotting activation and fibrin deposition in cell–mediated immune responses, it is suggested that the inhibitory effect on cell TF and PAI–2 expression might represent one of the mechanisms whereby OTA exerts its immunomodulatory activities.

Keywords: mycotoxins, tissue factor, PAI–2
Lipoprotein(a) Isoform Size Heterogeneity: Functional Implications for Plasminogen Activation

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Lipoprotein(a) [Lp(a)] is a unique lipoprotein consisting of a low density lipoprotein (LDL)–like moiety covalently linked to apolipoprotein(a) [apo(a)]. Apo(a) is homologous to the fibrinolytic proenzyme plasminogen. Indeed, apo(a) has been shown to inhibit fibrinolysis and elevated plasma concentrations of Lp(a) are associated with an increased risk for a variety of thrombotic disorders. Lp(a) exhibits isoform size heterogeneity in the population; differently-sized Lp(a) isoforms reflect varying number of copies (12 to >35) of repeated kringle IV (KIV) domains in apo(a). Low molecular weight isoforms of apo(a) (<22 KIV domains) have been implicated as an independent risk factor for cardiovascular disease. It has also been demonstrated that small isoforms of apo(a) bind to fibrin with increased affinity. Taken together, these studies suggest that the isoforms of apo(a) are functionally heterogeneous. We have undertaken the current study to investigate the effect of different apo(a) isoform sizes on the reactions of fibrinolysis. We have constructed a series of recombinant apo(a) [r–apo(a)] variants that represent greater than 90% of known apo(a) isoform sizes (12 to 30 KIV domains). A fluorescence-based plasminogen activation assay was employed to study the effect of apo(a) isoforms on plasminogen activation in the absence of plasmin-catalyzed positive feedback reactions, using soluble fibrin degradation products (FDPs) as the cofactor. An inverse trend was observed between the apo(a) isoform size and the extent of inhibition, with smaller isoforms inhibiting tPA-mediated Pg activation more than larger isoforms. Surface plasmon resonance was utilized to measure the binding of apo(a) isoforms to FDPs; it was found that smaller isoforms bound FDPs more readily than larger isoforms. This variability in affinity may at least partially account for the difference in inhibitory ability among the isoforms observed in the plasminogen activation assay. Additionally, the ability of apo(a) to inhibit the plasmin-mediated conversion of Glu–plasminogen to Lys–plasminogen, an important positive feedback mechanism that enhances the rate of plasminogen activation, was investigated. The results demonstrate that in the presence of apo(a), with either fibrin or FDPs as the cofactor, Glu–plasminogen to Lys–plasminogen conversion is attenuated. Furthermore, an increase in isoform size from 14 KIV domains to 23 KIV domains resulted in a decrease in the extent of inhibition observed, whereas a larger 27 KIV domain species was observed to be a less effective inhibitor of Glu–plasminogen to Lys–plasminogen conversion, indicating a biphasic inhibitory trend with respect to isoform size. Taken together these results clearly demonstrate that apo(a) isoforms are functionally heterogeneous with respect to their interaction with the plasminogen system.

Keywords: lipoprotein(a), plasminogen, fibrinolysis
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