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Review

Novel aspects and new roles for the serine protease plasmin

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Abstract. The serine protease plasmin is distributed throughout the human body in the form of the zymogen plasminogen. The plasminogen activation system is mostly recognized for its fibrinolytic activity but is also upregulated in chronic inflammatory diseases, including atherosclerosis and arthritis. Plasmin can bind to a variety of cells, including monocytes, through low-affinity binding sites and triggers aggregation of neutrophils, platelet degranulation and arachidonate release from endothelial cells. In monocytes, plasmin elicits full-scale proinflammatory activation, including lipid mediator release, chemotaxis and cytokine expression, as well as induction of other proinflammatory genes. The effects of plasmin are specific, require the active catalytic center and can be antagonized by lysine analogues, implying binding of the plasmin molecule to the cell membrane through its lysine binding sites. In view of the upregulation of the fibrinolytic genes in chronic inflammatory diseases, cell activation by plasmin is likely to play a major pathophysiological role, a view that is further supported by data from transgenic mice.

Key words. Plasmin; gene expression; transcription factors; serine protease; signaling; inflammation; monocytes.

Introduction

Serine proteases including plasmin are ubiquitously distributed throughout the human body in forms of zymogens [1] that may be activated under pathophysiological conditions. Apart from their pivotal role in the maintenance of haemostasis, these proteases may also transduce intracellular signals and modulate immune and inflammatory responses. The plasminogen/plasmin system consists of the inactive pro-enzyme plasminogen, which is transformed into the proteolytically active plasmin mainly by two plasminogen activators, tPA and uPA. While tPA plays a dominating role during fibrinolysis, uPA is an activator of the extracellular proteolysis, e.g. in inflammatory processes [2, 3]. In addition, plasminogen can be activated by kallikrein but also by an incompletely characterized plasminogen activator and, to a certain extent, by coagulation factors XIa und XIIa [4–7].

The functions of the serine protease plasmin in the process of fibrinolysis leading to lysis of the fibrin clot are well known [2, 3]. Yet for plasmin, with its broad substrate specificity, fibrin is not the only physiological substrate. Plasmin plays an important role during cell invasion and chemotaxis, as well as during tissue remodelling, which are essential during many pathological processes and require degradation of the extracellular matrix. Plasmin itself can directly degrade a few components of the extracellular matrix such as laminin and fibronectin, whereas other components are degradated by matrix metalloproteinases, which can be activated by plasmin. Thus, two proteolytic systems, the fibrinolytic system and the matrix metalloproteinases, degrade in concert the extracellular matrix [8]. The aforementioned effects of plasmin, which are mediated by its proteolytic activity, have been extensively reviewed elsewhere and will not be discussed here.

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Plasmin regulation

Plasminogen is predominantly synthesized in the liver, but plasminogen messenger RNA (mRNA) has been detected in a variety of other cell types and tissues [1]. Plasminogen is a glycoprotein consisting of 791 amino acids, with an apparent molecular weight of 92 kDa. The molecule can be divided into different structural domains; the 'preactivation peptide', five sequential kringle domains containing about 90 amino acids and a protease region with the catalytic triad His603, Asp646 and Ser741, which identify the molecule as a serine protease. The kringle domains contain the lysine binding sites, which are essential for specific recognition of fibrin, membrane binding sites and the physiological inhibitor α_2-antiplasmin.

Activation of the plasminogen molecule to the double-chain protease plasmin involves cleavage of the peptide bond Arg^{561}/Val^{562} between kringle 5 und the protease domain. The kringle structures compose the A-chain, which is connected to the B-chain through two disulfide bridges. The B-chain contains the serine protease region.

The activity of plasmin is tightly regulated by the plasminogen activators tPA and u-PA, as well as protease inhibitors. α_2-Antiplasmin is the principal endogenous plasmin inhibitor, which in the most rapid known protein-protein reaction finally covalently interacts in a multistep process with active plasmin, thereby leading to its inactivation. Accordingly, plasmin in the fluid phase is almost immediately inactivated by α_2-antiplasmin, yielding plasmin-α_2-antiplasmin complexes as a definite indicator of increased plasminogen activation. In contrast to plasmin in the fluid phase, membrane-bound plasmin is inaccessible to α_2-antiplasmin [9, 10]. Therefore, binding of plasminogen to fibrin and to a variety of cells controls not only the rate of the plasminogen activation and but also the susceptibility of plasmin to inactivation by inhibitors [10]. Fibrin stimulates the rate of tPA-mediated plasminogen activation by two orders of magnitude as a result of binding and colocalization of plasminogen and tPA to the fibrin surface [11]. Both uPA and tPA are able to bind to cells, leading to increased plasmin production and decreased inhibition of tPA by plasminogen activator inhibitor type 1 (PAI-1) [12].

Accordingly, the activation of plasminogen is dependent on the availability of the binding sites for plasminogen itself and for its activators. Binding of plasminogen is mediated by its lysine binding sites, specifically those located in the kringle 1 and 5 domains of the molecule. Efficient binding of plasminogen requires C-terminal lysines [10, 13, 14], which are subject to regulation. The availability of the plasmin/ogen binding sites on the surface of the cells and on fibrin is increased after treatment with proteases, which cleave protein substrates and expose new C-terminal lysines, e.g. thrombin and plasmin, or remodel the cell surface to enhance the accessibility to plasminogen binding sites, e.g. elastase and cathepsin G [10]. Opposite effects have basic carboxypeptidases (CpN and CpB), which can remove C-terminal lysines from cell surfaces and thereby decrease plasminogen binding to the cells [15]. Accordingly, in the mice partially or totally deficient in carboxypeptidase B, fibrinolysis was significantly enhanced in pulmonary clot lysis, and leukocyte migration was increased in thioglycollate-induced peritoneal inflammation [16]. Nonenzymatic regulation of the cell surface binding sites for plasmin has been also reported [10].

Activation of plasminogen in the presence of fibrin is also subject to regulation by low density lipoprotein (LDL), lipoprotein(a) [Lp(a)]. Elevated Lp(a) plasma levels constitute an independent risk factor for the development of atherosclerosis [17, 18]. In contrast to other LDL, Lp(a) contains an additional glycoprotein moiety called apolipoprotein(a) [apo(a)] which is strikingly similar to plasminogen. Lp(a) is able to bind to plasmin/ogen binding sites. Accordingly, Lp(a) competes with plasmin/ogen for cellular binding sites as well as for sites located on fibrin. This mechanism is believed to provide a functional basis for the atherogenic effects of Lp(a). For example, monocytic U937 cells bind plasminogen and Lp(a) with similar K_D. However, Lp(a) occupies only about 10% of the existing plasminogen binding sites, a fact that can be explained by the heterogeneity of the binding sites on a single cell type [18]. The apo(a) moiety of the Lp(a) molecule also interferes with plasmin generation; kinetic studies of plasminogen activation in the presence of different apo(a) isoforms have demonstrated interference of apo(a) with the complex consisting of tPA, plasminogen and fibrinogen, slowing down the turnover of the reaction [19, 20].

The regulation of proteins involved in plasmin activation also takes place at transcriptional and posttranscriptional levels [21]. For example, regulation of the expression of the plasminogen gene in hepatocytes by interleukin (IL)-6 has been described [22]. However, taking into consideration that plasminogen is ubiquitously distributed in human liquids and tissues with plasma levels of approximately 2.6 μM [23], it is not clear whether the up-regulation of the plasminogen expression by IL-6 is of any pathophysiological relevance. Autodegradation of plasmin catalysed by annexin II heterotetramer associated with fibrin clot might also contribute to regulation of plasmin activity in vivo [24].

Plasmin in pathophysiological processes

Plasmin is believed to play an important role in a number of physiological and pathophysiological processes. Different studies have clearly shown that plasmin, primarily in the cell-associated form, is important during ovulation,

migration of tumor and immune cells, during wound healing and possibly also during atherogenesis [25–31]. In line with experimental findings, plasminogen-deficient patients exhibit recurrent respiratory infections, vulvovaginitis and impaired wound healing. In addition, some patients with plasminogen deficiency develop ligneous conjunctivitis, pseudomembranous disease or hydrocephalus [1, 32].

Involvement in chronic inflammation

The pathophysiological importance of the plasmin/plasminogen system in classical inflammatory diseases such as arthritis or atherosclerosis has been well established by several clinical studies. Patients with rheumatoid arthritis exhibit increased levels of uPA in their synovial fluid [33] and synovial tissue [34]. In addition, expression of urokinase-type plasminogen activator receptor (uPAR) and PAI-1 is also increased in synovial tissue of rheumatoid arthritis patients [35]. Moreover, monocytes from these patients exhibit increased uPAR expression that might indirectly contribute to the enhanced plasminogen activation detected on monocytes from patients with rheumatoid arthritis [36, 37]. Indeed, patients also show an increase, although to a different extent, in the levels of plasmin-α_2-antiplasmin complexes in synovial fluid as well as in plasma [38, 39]. Increased plasmin production is not restricted solely to the pathological processes during rheumatoid joint diseases, but is also observed in other inflammatory joint diseases [39, 40]. In this setting, degradation of C1 inhibitor by plasmin might at least partially contribute to the proinflammatory effects of plasmin [41]. In the light of the proinflammatory effects of proteases, plasmin can indirectly affect the inflammatory process, at least in part, via degradation of this protease inhibitor.

The role of the fibrinolytic system was investigated in collagen-induced arthritis in both uPA- or tPA-deficient mice. The uPA$^{-/-}$ mice developed significantly milder disease, with cytokine levels correlating with the severity of the disease and particularly low IL-1β levels. In contrast, the disease was enhanced in tPA$^{-/-}$ mice, showing higher levels of fibrin deposition and cytokine production [42]. An explanation for these seemingly contradictory data might be the different functions of the plasminogen activators. Whereas tPA is important for plasmin-mediated dissolution of fibrin clots and exerts an enhanced effect upon binding to fibrin, uPA possesses no fibrin-related efficacy and generates plasmin at the cell surface, suggesting a different biological role. Thus, reduced tPA activity, but not the uPA activity, leads to fibrin accumulation, which might stimulate cytokine production and potentiate the disease [43]. On the other hand, uPA activity is correlated with the degradation of cartilage associated with elevated IL-1 or tumor necrosis factor (TNF) α [33, 44, 45], linking cell membrane-bound plasmin generation to production of cytokines in arthritis. However, evidence that the proinflammatory effects of uPA in this model might be mediated by plasmin generated in close proximity to cytokine-producing cells remains to be shown.

Involvement of plasmin in the inflammatory process has also been demonstrated in plasminogen-deficient mice. In thioglycolate-induced peritoneal inflammation the kinetics and extent of early neutrophil recruitment to the inflamed peritoneal cavity was similar to that of wild-type mice. Recruitment of lymphocytes and especially of monocytes and macrophages was, however, severely compromised [46, 47], indicating that macrophages rely directly or indirectly on the plasmin/ogen system for migration to the site of inflammation. In agreement with these observations, plasminogen knockout mice exhibit impaired wound healing [27].

If membrane-bound plasmin is to be considered as a therapeutic target, novel approaches could possibly be developed for the treatment of chronic inflammatory diseases. Thus, inhibition of plasmin by adenoviral vectors encoding an engineered cell surface-targeted plasmin inhibitor seems to reduce synovial fibroblast-dependent cartilage degradation and invasion in rheumatoid arthritis [48], as well as restenosis in the ballon catheter-injured rat carotid artery model [49].

A role for the plasminogen activation system in atherosclerosis

An inflammatory reaction in the vessel wall is an essential component of the atherosclerotic process [50]. Despite the fact that it was already postulated a long time ago that the fibrinolytic activity is upregulated in the human atherosclerotic lesions [28, 51–55], only recently, with the help of Northern blotting and in situ hybridization, has the increased expression of fibrinolytic genes in the altered atherosclerotic aorta been confirmed [29, 56]. The increased gene expression in histological preparations is colocalized with the inflammatory infiltrate, with migrating vascular smooth muscle cells and CD68-positive macrophages [29]. Presently it is assumed that a correlation exists between local plasmin production and the development of abdominal aortic aneurysms. It has been suggested that degradation of the extracellular matrix by newly generated plasmin together with the activation of latent metalloproteinases contributes, directly and indirectly, to the specific clinical symptoms and to proteolytic degradation of the vessel wall [29].

Additional information about the involvement of fibrinolytic genes in the development of atherosclerosis was obtained from experiments with transgenic animals. In a mouse model of transplant artherosclerosis, carotid vessels from wild-type mice exhibit suppressed smooth

muscle cell proliferation and migration into the intima, reduced intima necrosis as well as diminished inflammation of adventitia and intima when transplanted into $Plg^{-/-}$ recipient mice [57]. In addition, the neointimal growth in the $Plg^{-/-}$ recipient mice was significantly lower than that in wild-type recipient mice. Whereas the $Plg^{-/-}$ mice exhibit reduced formation of atherosclerotic lesions, the protective effect was not observed in double-knockout $Plg^{-/-}$ and $apoE^{-/-}$ mice on a high fat diet, possibly suggesting a dichotomy of plasmin/plasminogen effects [47]. A study investigating the development of atherosclerosis in mice deficient in both, apolipoprotein E (apoE) and plasminogen demonstrated that the absence of plasminogen accelerates lesion development and amplifies the atherosclerotic process in the context of the ongoing atherogenesis [32, 58]. At present it is difficult to evaluate the significance of the conflicting data from the $apoE^{-/-}$ mouse model because the model itself is subject to some criticism as far as its relevance for human atherosclerosis is concerned. Nevertheless, these two studies suggest complex effects of plasmin, which might depend on the type of experimental atherosclerosis. It is also possible that the size of the lesion itself might not be a proper parameter for the development of atherosclerosis. Thus, the increased level of PAI-1 in atheromas and in the vessel walls is likely to potentiate formation of vulnerable atheroma plaques, which are smaller and contain fewer cells highly laden with lipids. Such plaques have thin fibrous caps and are prone to rupture [59].

In humans, increased severity of artherosclerotic lesions in coronary arteries is positively correlated with the expression of the specific plasminogen activator uPA. Data from studies with mice deficient in fibrinolytic genes also confirmed a role for uPA and plasminogen, but not for tPA in smooth muscle cell migration and neointima formation after vascular injury [60, 61]. These and additional data from human coronary artery specimens [56] indicate that the uPA/plasminogen system might be causally related to atherogenesis.

In cardiac injury early cardiac wound healing requires recruitment of inflammatory cells to the site of lesion followed by complex processes leading to myocardial scar formation. In a study with plasminogen-deficient and wild-type mice it was shown that disruption of the plasminogen gene delayed cardiac wound healing by at least 5 weeks after experimental myocardial infarction [32, 62, 63], supporting the view that plasminogen is involved in different wound-healing processes.

Plasmin and cancer

There is a large body of evidence implying a critical role for plasminogen activation in tumor cell invasiveness and metastasis [26, 64]. Constitutive overexpression of the uPA gene is a characteristic of malignant progression, leading to high levels of receptor-bound urokinase that triggers subsequent plasminogen activation. Membrane-bound plasmin that is protected from circulating inhibitors degrades proteins of the extracellular matrix such as laminin and fibronectin and activates various matrix metalloproteinases, which further contribute to extracellular matrix degradation. Plasmin generated at the surface of tumor cells is, therefore, considered a key event in tumor invasion and metastasis. Elevated levels of uPA, uPAR and PAI-1 appear to be markers of poor prognosis in colorectal, breast and other tumors [32, 65]. However, a number of experiments with plasminogen-deficient mice have shown that tumors also rely on other proteolytic pathways for growth and metastasis. For example, the primary tumors of Lewis lung carcinoma formed in plasminogen-deficient mice were smaller, less hemorrhagic and exhibited less skin ulceration. Further, dissemination to the regional lymph nodes was delayed in plasminogen-deficient mice compared with wild-type control animals. However, there were no differences in pulmonary metastasis. In a mouse mammary adenocarcinoma model, there were no differences in tumor development between plasminogen-deficient and wild-type mice; the amount of lung metastasis was, however, significantly reduced in mice with plasminogen gene disruption [32]. Likewise, studies on the plasminogen system in vivo provided evidence for the requirement of uPAR, PAI-1 and probably uPA for angiogenesis, which is an integral part of tumor growth and invasion. However, plasmin is also the source of the anti-angiogenic angiostatin, which makes the regulation of the angiogenesis by the fibrinolytic system a very complicated process [66]. In the light of this complexity, further efforts will be required to elucidate, for example, the regulatory mechanisms involved in the uPA/plasminogen system. A detailed understanding of these processes might finally allow the pinpointing of therapeutic targets to fight tumor progression and metastasis.

Pathophysiologal aspects of plasmin in the central nervous system

The liver is the main source of plasminogen; however, plasminogen is also produced in brain tissue, including the hippocampus [1, 32, 67]. Experiments with plasminogen-deficient mice implicate plasmin in promoting hippocampal excitoxic neuronal death, probably through the disruption of neuron-laminin interactions [32]. Consistent with these findings, excitotoxin-mediated neuronal cell death was shown to be dependent on tPA, suggesting a possible role for plasmin in neurodegeneration [68].

On the other hand, a protective role for plasmin has been proposed in the development of Alzheimer's disease.

Brain tissue from patients with Alzheimer's disease contains reduced levels of plasmin. This may be causally very important, because plasmin that is associated with the lipid rafts of cultured hippocampal neurons cleaves the amyloid precusor protein, reducing amyloid plaque formation, an early event in Alzheimer's disease [69]. Together, these findings suggest that diminuished brain plasmin levels might be one of the causes of amyloid plaque formation rather than its consequence. Therefore, brain plasmin may possibly play a preventive role in amyloid precursor protein amyloidogenesis.

A further aspect, of yet unclear significance, concerns recent observations showing that a distinct isoform of the prion protein can stimulate tPA- but not uPA-mediated activation of plasmin, which might point to a role of plasmin the pathogenesis of the transmissible spongioform encephalopathies [12, 70].

These examples compiled from a wealth of data implicate a significant role of the plasmin system in a variety of human disorders. In most cases, the detailed mechanisms of the plasmin-mediated effects still remain to be elucidated.

Plasmin-induced cellular effects

Most of the plasmin-mediated effects are due to the proteolytic cleavage of a variety of extracellular proteins: fibrin, laminin, fibronectin, von Willebrand factor, thrombospondin and proteolytic activation of matrix metalloproteinases, growth factors and chemokines [10, 71–74]. These effects are mediated by substrates cleaved by plasmin and will not be reviewed here.

Generation of plasmin in close proximity to the cell surface raises the question about putative auto- or paracrine effects of plasmin on these cells. Until recently, the pericellular effects of plasmin have been mainly regarded in terms of membrane-associated fibrinolytic or proteolytic activity [3, 10, 75–77]. Plasmin can, however, trigger profound functional changes in a number of cells. The changes observed imply receptor-mediated signaling. Only recently another serine protease, thrombin, was shown to activate a number of cells via proteolytic cleavage of the so-called protease-activated receptors (PARs) that belong to the family of seven transmembrane domain receptors [78–80]. Meanwhile some other proteases have been shown to activate cells via PARs or other cellular receptors [79, 81]. It is intriguing to speculate that plasmin might exert its effect, at least in part, through a similar mechanism.

Cellular binding sites for plasmin

The plasminogen binding sites at the cell surface are heterogeneous, and their identity and especially their functional relevance is obscure. All blood cells, except of red blood cells, express binding sites for plasminogen, which could bind plasmin as well [3]. Typical characteristics of the plasmin binding sites are their broad distribution, low affinities with K_D values in the range of 0.1–2.0 μM, and high density (B_{max} about $0.4–5.0 \times 10^5$/cell). Lys-plasminogen, a short form of plasminogen that can be generated by plasmin, and plasmin itself are frequently observed to have lower K_D values [82].

Tumor cells, including monocytic cell lines, display particularly high Bmax values, which are one to three orders of magnitude higher than those for other cells. There are many indications for heterogenic plasmin/plasminogen receptors, but in the receptor binding studies, at least concerning the K_D values, often only a single class of binding proteins is found. However, numerous proteins, such as α-enolase [83, 84], annexin II und TIP49a on the monocytic U937 cells [85], annexin II on endothelial cells, macrophages and monocytes [86, 87], gp330 on kidney epithelial cells [88] and several others [65] have been identified as plasmin/ogen binding sites. Occasionally these binding sites were named receptors, mostly in terms of membrane-associated proteolytic activity without signal-transducing function. Some of these proteins already expose lysine, to which plasmin can bind. Some receptors have to be cleaved before the lysine residue is exposed and can bind plasminogen. Recently, Ranson and Andronicos reviewed in great detail the different plasmin-binding cell surface molecules, but mainly with respect to the plasmin-mediated proteolytic activity at the surface of tumor cells [65]. The identity of the plasmin binding site, which transduces the transmembrane signaling of plasmin, has not yet been identified [10, 75, 76].

Recently, the thrombin receptor PAR1 was implicated in the plasmin-induced expression of the Cyr61 gene in fibroblasts. Its signaling leads to a rapid and transient activation of ERK1/2 followed by the expression of Cyr61 [89]. Interesting, but also confusing is the fact that although the same cells demonstrate an increase in intracellular Ca^{2+} after stimulation of PAR1 with thrombin, plasmin failed to elicit any Ca^{2+} response through supposedly the same receptor. However, based on peptide studies in vitro, as well as in vivo studies in PAR1-expressing cells, other authors have demonstrated that plasmin desensitizes PAR1 by cleavage at three arginine and lysine residues located at the C-terminal side of the tethered region and N-terminal to ligand binding sites [90]. Others, using recombinant extracellular segments of PARs as well as analysis by MALDI mass spectrometry and surface plasmon resonance, similarly concluded that plasmin would disable PAR1 and PAR2 [91]. In our own experiments with human monocytes, we could not detect any plasmin-mediated activation via PAR1, PAR2 or PAR3, whereas PAR4 is definitively not expressed by human monocytes [80].

Plasmin-induced cell activation

In human platelets plasmin triggers a biphasic reaction. Low plasmin concentrations (< 1.0 CU/ml) inhibit the aggregation followed by a number of different agonists, such as thrombin, collagen and ionophore A23187. Already at concentrations as low as 0.1 – 0.5 CU/ml, a full blockade was observed. In addition, plasmin inhibits, at least partially, mobilization of arachidonic acid from phospholipids [92]. At variance to low concentration, at levels ≥ 1.0 CU/ml plasmin induces aggregation of platelets. In this case, the signal transduction pathway apparently involves protein kinase C and phopholipase C. The protein kinases activated phosphorylate proteins with molecular weights of 20 and 47 kDa, which, however, have not been identified [93]. Others have observed phosphorylation of tyrosine residues of other proteins [94]. Plasmin exerts an extremely complex effect on the intracellular Ca^{2+} levels in platelets. On the one hand, plasmin triggers release Ca^{2+} from intracellular stores; on the other hand, this signal appears to be superimposed by a tyrosine kinase-dependent increase in Ca^{2+} turnover. As a result, a gradual, much slower elevation of intracellular Ca^{2+} is observed [93, 94]. The plasmin-induced shape changes in platelets are additionally regulated by biphasic reactions, Ca^{2+}-dependent and Ca^{2+}-independent Rho kinase-dependent pathways; both pathways are inhibited by cyclic AMP (cAMP)-elevating agents [95].

In contrast to platelets, in bovine endothelial cells plasmin stimulates the arachidonic acid cascade, which leads to activation of prostacyclin biosynthesis through a phospholipase C-independent mechanism. Similar to platelets, biphasic activation mechanisms have been observed in these cells. In the early phase of stimulation the release of arachidonic acid is Ca^{2+} dependent and pertussis toxin sensitive, pointing to receptor-mediated signaling. This assumption is supported by the fact that this process is dependent on the binding of plasmin via its lysine binding sites and the presence of the catalytic domain. In contrast, the late-phase activation process is G-protein-independent and requires only the catalytic center [96], suggesting, probably, unspecific proteolytic activation of the cell. More recent studies with this cell type revealed that plasmin but not plasminogen binds to integrin $\alpha v\beta_3$ through its kringle domains and triggers plasmin-induced stress fiber formation and migration of endothelial cells, a mechanism relevant for the process of angiogenesis. This activity of plasmin required both an interaction with $\alpha v\beta_3$ and catalytic activity of plasmin [97]. However, it is not clear whether $\alpha v\beta_3$ integrin transduces plasmin signaling. It was suggested that $\alpha v\beta_3$ might increase the plasmin concentration on the cell surface and, thus, facilitate activation of a putative plasmin receptor through proteolytic cleavage. These findings could be very important, because $\alpha v\beta_3$ is also the binding site for angiostatin, the antiangiogenic proteolytic fragment of plasmin. Indeed, blockade of plasmin-mediated signaling in endothelial cells could provide the molecular basis for the well-known antiangiogenic effects conveyed by angiostatin.

In human umbilical cord endothelial cells plasmin stimulates the adherence of neutrophils [98]. The adherence can be inhibited by pretreatment either with monoclonal antibodies against P-selectin, or with anti-CD18 antibodies. Interestingly, this process is dependent on biosynthesis of the platelet-activating factor (PAF). Plasmin-induced synthesis of PAF in endothelial cells correlated with the degree of adhesion of neutrophil leukocytes and can be antagonized by pretreatment with a PAF antagonist [98]. It was therefore assumed that plasmin-induced biosynthesis of PAF in human endothelial cells leads to upregulation of P-selectin expression. These data suggest that plasmin would render the endothelial surface proadhesive for neutrophils and may favor a PAF-mediated increase in vascular permeability.

Plasmin induces aggregation of neutrophils, which again is dependent on lysine binding sites and the active catalytic center [99]. It is possible that the adhesion molecule CD18 is involved in the aggregation. CD18 is activated in neutrophils after stimulation with plasmin [100]. Expression of two types of adhesion molecules on endothelial cells and neutrophils may provide the basis of the CD18-P-selectin-mediated adhesion of neutrophils to endothelial cells. In fact, this process represents the initial phase of the inflammatory reaction.

Plasmin has also been implicated in the proliferation of hepatocytes in primary culture. The cell proliferation could be inhibited by the lysine analogue tranexamic acid and PASI-535, an active-center-directed inhibitor of plasmin, again pointing to a plasmin-dependent signaling mechanism [101]. Similarly, in bovine retinal pericytes plasmin treatment led to increased DNA synthesis [102]. These data suggest that plasmin, at least in certain cell types, might be able to exert mitogenic activity.

These examples demonstrate a variety of diverse effects of plasmin on various cell types. The cellular effects of plasmin are obviously heterogeneous and not fully characterized. More detailed data on plasmin-induced intracellular signaling have been obtained in experiments with human peripheral blood monocytes.

Plasmin-mediated biosynthesis of lipid mediators by monocytes

Monocytes and macrophages, as antigen-presenting cells as well as direct effector and cytokine-producing cells, play a key role in the inflammatory reaction [103]. However, macrophages can proliferate in tissues to a small extent only; the accumulation of mononuclear phagocytes at the site of inflammation is due to the directed migration of monocytes from peripheral blood [104]. In chronic inflammation [105] and especially in the arte-

riosclerosis-associated inflammatory lesions of the vessel wall [50], monocytes and macrophages play a predominant pathophysiological role.

Monocytes and monocytic cell lines carry binding sites for plasmin/ogen and uPA on their surface, which were first described by Plow et al. [3, 77]. Monocytes are also able to generate PAI-1 und -2. As a result, this cell type has diverse mechanisms for controlling the fibrinolytic activity close to the cell surface [77, 106, 107]. The importance of the plasmin/ogen system for monocytes is further emphasized by the findings that activation with either LPS or cytokines leads to increased expression of the uPAR [108] and, as a result to increased plasmin production. During chemotactic migration in a N-formyl-methionyl-leucyl-phenylalanine (FMLP) gradient, monocytes polarize their uPARs at the migration front [30]. Interestingly, for tissue invasion monocytes require uPA bound to its receptor. Therefore, it is assumed that monocytes enter into interstitial tissues with the help of the proteolytic activity of plasmin [31].

For the optimal function of monocytes as antigen-presenting and effector cells they have to be activated by T cell lymphokines, for example interferon-γ, or bacterial products such as endotoxic lipopolysaccharide (LPS). Activation means the induction or amplification of endogenous or exogenous processes, which secure the optimal immune reaction. Autocrine stimulation plays an important role in this process [109]. Recent data suggest that plasmin might act as a potent and specific activator of human monocytes.

The first indication that plasmin might be involved in the production of the lipid mediators came from experiments with coagulating whole human blood. Contact-mediated activation of the intrinsic coagulation cascade in human blood triggers the biosynthesis of cysteinyl-leukotrienes und leukotriene B_4, which are generated via the 5-lipoxygenase pathway [110, 111]. Thrombin generated by the coagulation cascade however, stimulates only the cyclooxygenase pathway and, thus, the synthesis of prostanoids, but had no effect on the 5-lipoxygenase pathway [112]. Unexpectedly, supplementation experiments with autologous cells showed that contact-mediated activation of the 5-lipoxygenase pathway in coagulated human whole blood takes place in monocytes, but not in neutrophils, suggesting a cell-specific activation mechanism [113]. Monocyte activation in contact-stimulated blood could be inhibited by different lysine analogues, α_2-antiplasmin and aprotinin, and it was amplified by the plasminogen activators urokinase and streptokinase. In addition, a specific monoclonal antibody which prevents the proteolytic activation of plasminogen to plasmin, completely blocked the monocyte-mediated release of leukotrienes. Hence, all the data pointed to plasmin, which is produced in small amounts during contact-triggered intrinsic fibrinolysis, as an activator of the 5-lipoxygenase pathway in monocytes. The effect was specific for plasmin, because a number of other proteases such as coagulation factors Va, Xa, the serine proteases thrombin, α-chymotrypsin, human neutrophil elastase and cathepsin G were unable to trigger a selective activation of 5-lipoxygenase pathway [114–116]. Plasmin-induced leukotriene biosynthesis was not mimicked by plasminogen, and in line with that, catalytic center-blocked plasmin was unable to elicit leukotriene formation by monocytes. These data revealed some preconditions for the plasmin-induced effects, i.e. binding of plasmin to the cell surface through its lysine binding sites and the proteolytic activity of the molecule. Further experiments with isolated cells confirmed that plasmin-mediated activation of the 5-lipoxygenase pathway in monocytes but not in neutrophils, which similarly possess all the enzymes necessary to generate leukotrienes via the 5-lipoxygenase pathway [116]. Evidence for the pathophysiological relevance of these findings came from experiments showing selective activation of leukotriene biosynthesis both in clamped umbilical vessel segments still filled with autologous blood immediately after delivery as well as in fresh human thrombus specimens obtained by therapeutic thrombectomy [113].

Plasmin-induced migration of monocytes

Chemotaxis, which is cell migration towards a chemoattractant, is essential for the inflammatory response during which monocytes migrate from the bloodstream to the sites of inflammation. Plasmin induced a chemotactic response in monocytes with only insignificant effects on the monocyte chemokinesis, i. e. random migration [117]. Plasmin induced a potent monocyte chemotaxis and actin polymerization that was comparable to that induced by the standard chemoattractant FMLP. The requirements for the plasmin-mediated monocyte chemotaxis were membrane binding through lysine binding sites and the presence of the intact catalytic domain; indeed, the effect of plasmin could be antagonized by catalytically inactivated plasmin, suggesting a selective receptor-mediated event. Plasmin was chemotactic only for monocytes; neutrophils migrating to FMLP failed to respond to plasmin, again indicating a cell-specific effect [117].

Using pharmacological inhibitors it was demonstrated that plasmin activates the monocyte chemotaxis through a pertussis toxin-sensitive G protein and protein kinase C. Unlike other chemoattractants, plasmin does not induce an increase in intracellular Ca^{2+}; accordingly, no increase in inositol-1,4,5-triphosphate could be detected [116]. A similar unexpected lack of intracellular Ca^{2+} increases was described in plasmin-stimulated fibroblasts [89]. On the other hand, plasmin induced an increase in cGMP, which was absolutely essential for monocyte chemotaxis; the inhibition of cytosolic guanylate cyclase leads to a

concentration-dependent reduction of the chemotactic response. This effect could be antagonized by stable cGMP analogues. Inhibition of the cGMP-dependent protein kinase also decreased the monocyte chemotaxis, indicating the critical role of G-kinase-dependent protein phosphorylation for plasmin-induced cell migration [117].

Interestingly, the effect of plasmin on monocytes could be mimicked by Lp(a). Atherogenic Lp(a), which contains apo(a), a protein with high structural homology to the plasminogen molecule, similarly triggered chemotaxis in monocytes [118]. The signal transduction pathway activated by Lp(a) was similar to that of plasmin and includes a pertussis toxin sensitive G protein, protein kinase C, soluble guanylate cyclase and cGMP-dependent kinase(s). Unlike Lp(a), other low-density lipoprotein (LDL) particles did not induce monocyte chemotaxis. Treatment of monocytes with recombinant apo(a) revealed that it is the apo(a) moiety of Lp(a) that is responsible for monocyte activation [118]. This remarkable resemblance of the signaling between plasmin and apo(a) suggests that only a small part of the plasmin binding sites which can also bind Lp(a) might be responsible for transduction of the transmembrane signal in monocytes.

Plasmin-induced gene expression in monocytes

Activation of monocytes during physiological and pathophysiological reactions results in the expression of proinflammatory genes. Activated monocytes are the source of over 100 mediators of inflammation. Best studied is regulation of the expression of the cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). The production of these cytokines is regulated at multiple levels: at the level of transcription factors, translation and processing of precursors. There is evidence that TNF-α production in Kupffer cells and in rat peritoneal macrophages can be modulated by cGMP [119, 120]. In human monocytes, sodium nitroprusside stimulates cGMP formation and the release of TNF-α, but not IL-1β [121]. Expression of genes during cell activation is regulated by transcription factors such as activator protein 1 (AP-1) and nuclear factor kappaB (NF-κB) [122–124]. NF-κB binding sites are found in promoter regions of numerous inducible genes, for example, IL-1, IL-6, TNF-α, monocyte chemoattractant protein 1 (MCP-1) und tissue factor (TF). It is accepted that NF-κB plays the central role in coordination of gene expression during activation of monocytes and macrophages [124, 125].

Like endotoxic LPS, plasmin induces expression of the cytokines TNF-α, IL-1α and β, the chemokine MCP-1 as well as TF and CD40 in purified monocytes [126, 127]. As plasmin-induced chemotaxis and leukotriene release, gene expression is inhibited by a lysine analogue, indicating that plasmin binds monocytes via lysine binding sites. Plasmin-induced gene expression was also inhibited in the presence of the plasmin inhibitor aprotinin. The kinetics of the cytokine and tissue factor production by plasmin-stimulated monocytes were slower than in LPS-stimulated cells. But the amount of cytokines released was similar in plasmin as well as in LPS-treated cells [126].

Both cytokines and TF belong to the so-called 'early-immediate genes', which are rapidly expressed upon activation of transcription factors such as NF-κB and AP-1 [122, 128–130]. In fact, within 1 h of plasmin stimulation activation of NF-κB reaches a maximum. The transcription complexes contained the proteins p50, p65 und c-Rel. Rapid induction of NF-κB followed activation of inhibitor kappaB (IκB) kinase, IKKβ, leading to phosphorylation and degradation of two NF-κB inhibitors, namely IκBα and, unexpectedly, p105. Interestingly, plasmin triggered full degradation of p105 rather than its processing to p50. Activation of IKKβ, but not of IKKα, rapidly after stimulation indicates a direct effect of plasmin on NF-κB activation [126].

Optimal gene expression requires simultaneous activation of several transcription factors. Indeed, plasmin is able to activate several signaling cascades in monocytes which cooperate in the induction of proinflammatory genes. Within minutes plasmin induces phosphorylation and thereby activation of mitogen-activated protein kinase (MAPK) kinase 3/6, followed by p38 MAPK [127]. In addition, plasmin triggers the janus kinase (JAK)/signal transducer und activator of transcription (STAT) signaling pathway. Among four known JAK kinases, JAK1, JAK2, JAK2 and TYK2, only JAK1 was phosphorylated after plasmin treatment. Phosphorylation already occurs after 5 min, again indicating a direct effect of plasmin on this signaling cascade. Activated p38 and JAK1 phosphorylated, and thereby activated, the transcription factors STAT1 and STAT3 on tyrosine and serine residues. This double phosphorylation of STAT proteins leads to translocation to the nucleus and binding to DNA containing STAT response elements. Using gel shift assays, it can be demonstrated that within 20 min after stimulation of monocytes with plasmin, the STAT/DNA complexes contain more STAT3 and fewer STAT1 dimers. Inhibition of JAK1 and p38 kinases results in impaired phosphorylation and DNA binding of STATs [127].

As a result of p38 phosphorylation, another transcription factor, AP-1, essential for the expression of cytokine and TF genes, is activated in monocytes after treatment with plasmin [126, 127]. Activated AP-1 contains phosphorylated ATF-2 and c-Jun. Inhibition of p38 kinase, but not of JAK or ERK1/2, resulted in a total blockade of AP-1 activation. Plasmin-mediated activation of p38 MAPK and JAK/STAT pathways is indispensable for the expression of MCP-1 und CD40 in human monocytes. Plasmin-induced expression of MCP-1 and CD40 could be inhib-

ited by pharmacological inhibition of JAK and p38, while inhibition of ERK1/2 with U0126 did not affect gene expression [127]. These data indicate that in monocytes plasmin triggers multiple signaling pathways which converge leading to full-scale monocyte activation, ranging from stimulation of biosynthesis of lipid mediators to gene expression.

Interestingly, plasmin-induced chemotaxis and cytokine expression in monocytes can be inhibited by the peroxisome proliferator-activated receptor gamma (PPARγ) activators 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and ciglitazone, but not by the PPARα activator clofibric acid [131]. Pretreatment of monocytes with 15d-PGJ_2 and ciglitazone resulted in inhibition of plasmin-induced NF-κB and AP-1 activation. However, it is not clear whether the effects observed are due to activation of PPARγ in monocytes. Another activator of PPARγ, BRL49653, had no effect on plasmin-induced monocyte activation [un-

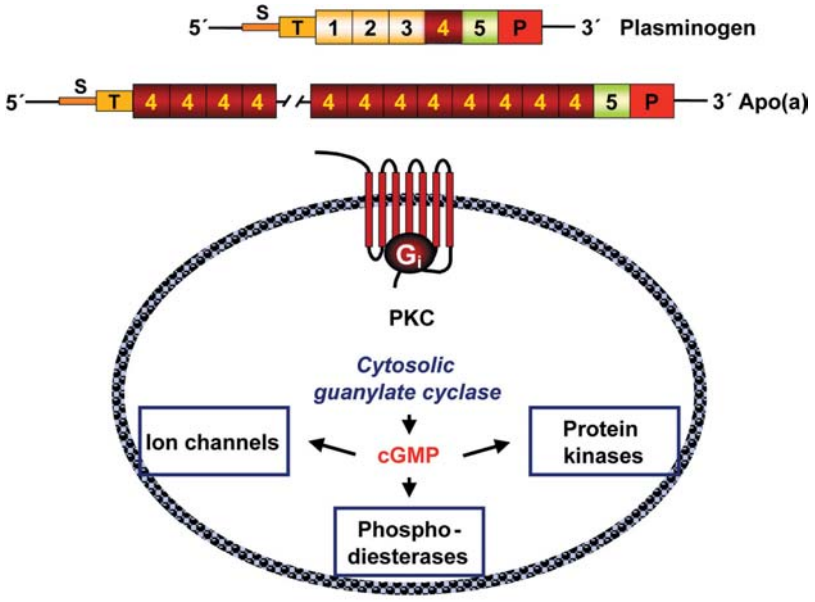

Figure 1. Sequence homology between plasminogen and the apolipoprotein(a) moiety of lipoprotein(a). In human peripheral monocytes plasmin as well as lipoprotein(a) trigger activation of protein kinase C (PKC) and the soluble guanylate cyclase, followed by release of cGMP, which are essential for the plasmin and lipoprotein(a)-induced monocyte chemotaxis.

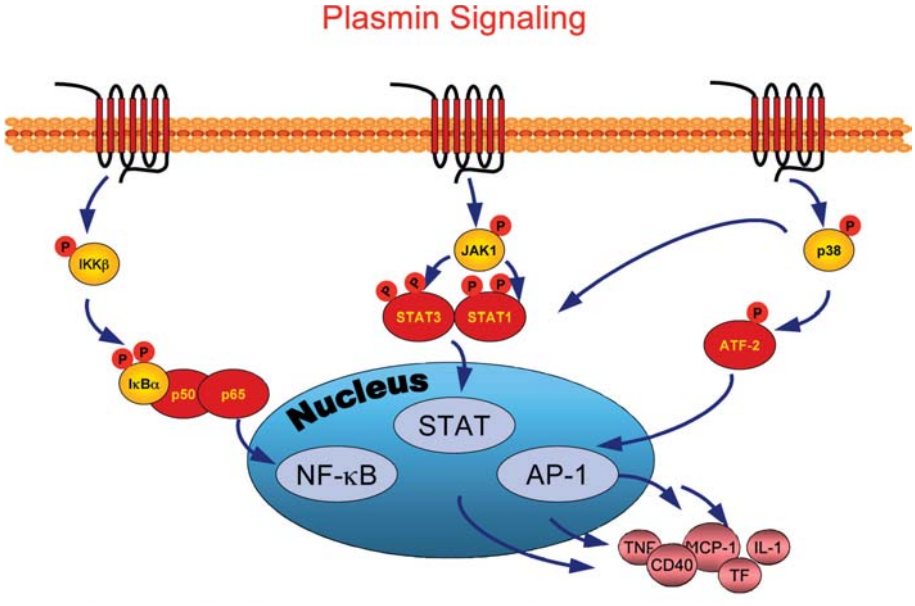

Figure 2. Plasmin-induced phosphorylation/activation of NF-κB, JAK/STAT and p38/AP-1 pathways, leading to the expression of TNF-α, IL-1α, IL-1β, MCP-1, CD40 and tissue factor genes.

published observations]. The effects of 15d-PGJ_2 and ciglitazone may be due to their effects on other signaling cascades. 15d-PGJ_2, for example, is known to directly affect IKK [132, 133]. Further, ciglitazone inhibited the p38 MAPK activation in plasmin-treated monocytes, which is essential for both monocyte chemotaxis and cytokine expression in plasmin-stimulated monocytes [131].

Conclusion

A substantial body of evidence indicates that the serine protease plasmin is a potent activator of human monocytes and a number of other cells. In monocytes plasmin elicits full-blown proinflammatory activation encompassing lipid mediator release, chemotaxis and induction of cytokines and other proinflammatory genes. Despite the fact that the information on plasmin-induced cellular effects is still rudimentary, some common features emerge. Thus, cell activation is dependent on the binding of the plasmin molecule via its lysine binding sites as well as on the intact catalytic center of plasmin, indicating proteolytic cell activation. Cell activation occurs through a yet unidentified receptor which is specific for plasmin and that, at least in monocytes, is not activated by other proteases, such as factors Va and Xa, the serine proteases thrombin, α-chymotrypsin, human neutrophil elastase or cathepsin G.

The data discussed reveal novel aspects of plasmin function in addition to its established role in fibrinolysis. Further studies will be necessary to delineate physiological relevance and analyse in greater detail the mechanisms of plasmin-mediated cell activation, which may ultimately allow identification of novel therapeutic targets.

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