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Review

Regulation and interactions in the activation of cell-associated plasminogen

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Abstract. The main components in plasminogen activation include plasminogen, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), and plasminogen activator inhibitors-1 and -2 (PAI-1, PAI-2). These components are subject to extensive regulation and interactions with for example, pericellular adhesion molecules. Although uPA and tPA are quite similar in structure and have common inhibitors and physiological substrates, their physiological roles are distinct. Traditionally, the role of tPA has been in fibrinolysis and that of uPA in cell migration, especially in cancer cells. Recently several targets for tPA/plasmin have been found in neuronal tissues. The functional role of the PAIs is no longer simply to inhibit overexpressed plasminogen activators, and PAI-2 has an unidentified role in the regulation of cell death.

Key words. Plasminogen; serpins; tPA; uPA; glucocorticoids; integrins; cell migration.

Introduction

The end product of the plasminogen activation cascade, plasmin, can cleave extracellular matrix proteins, activate proteinases and deliver growth factors. Based on its proteolytic capacity urokinase plasminogen activator (uPA)-activated plasmin is thought to be important in cancer cell invasion and tissue plasminogen activator (tPA)-activated plasmin in fibrinolysis in the vasculature. In addition, tPA plays a role in neuronal plasticity, in memory, learning and stress activities in the brain. Within the last decade new roles have emerged for plasminogen activator inhibitors (PAIs), which as 'jokers' can have additional functions independent of or depending on their inhibitory capacity. The complex scene of PAI-1 includes integrins, their extracellular matrix ligands and endocytosis by low-density lipoprotein (LDL) receptor-related protein (LRP).

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Plasminogen

Plasminogen (fig. 1) is a single-chain glycoprotein with a molecular mass of ~ 92 kDa [1, 2]. Plasminogen is synthesized mainly in the liver [3], and this inactive form circulates in the vasculature but is also found ubiquitously in other body fluids. Secreted plasminogen is a precursor that is cleaved by plasminogen activators at a single site, the Agr_{561}-Val_{562} bond [4], to yield a two-chain plasmin held together by two disulfide bonds [2]. The structural features of the plasminogen molecule [5] include an N-terminal (A-chain) component, which has a pre-activation peptide (from 1 to 77), followed by five tandem structures called kringle domains. Kringle domains participate in binding to fibrin and to the cell surface [6]. In the B-chain (carboxyl-terminal region) lies the catalytic domain with the His-Asp-Ser characteristic triad of serine proteinases [6].

The proenzyme plasminogen exists in the circulation with Glu (glutamic acid) amino acid in its NH_2-terminus,

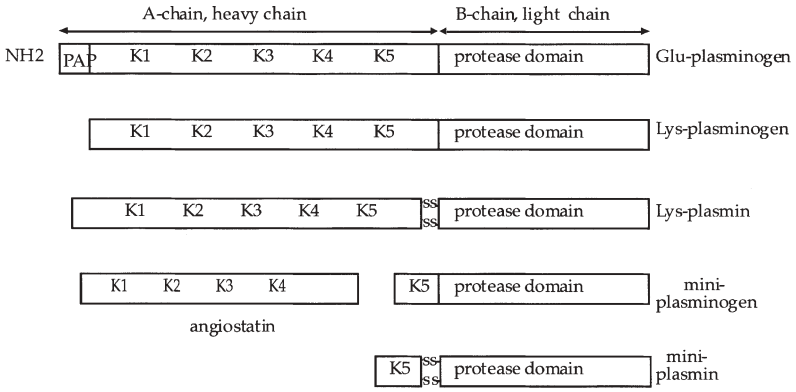

Figure 1. Schematic diagram of plasminogen and its cleavage products. PAP, pre-activation peptide; K, kringle; Glu, glutamic acid; Lys, lysine.

and cleavage of the N-terminal peptide produces Lys-terminal plasminogen, which is more readily activated by plasminogen activators [7]. Trypsin can activate plasminogen by activating pro-urokinase [8]. Plasminogen can also be cleaved with elastase. Elastase cleavage of plasminogen yields two fragments: angiostatin, including kringles 1–4, and miniplasmin, consisting of the serine protease domain and kringle 5 [9]. Amphoterin and annexin II bind to plasminogen [10, 11]. In addition, plasminogen binds to platelet integrin $\alpha_{IIb}\beta_3$ [12], whereas angiostatin and plasmin (not plasminogen) interact with $\alpha_v\beta_3$ integrin [13, 14]. Plasminogen can be converted in a cell membrane-dependent reaction to angiostatin, which has been shown to inhibit angiogenesis [13].

tPA

The messenger RNA (mRNA) for tPA is ~2.7 kb and encodes a glycoprotein with a molecular mass of ~70 kDa [15, 16]. tPA protein is secreted as a single-chain glycoprotein. Similarly to uPA, tPA can be cleaved by plasmin to a two-chain form held together by a single disulfide bond. The tertiary structure of tPA differs from that of uPA. The N-terminal (A-chain or heavy-chain) region has a finger domain followed by a growth factor domain, and two kringle domains. The carboxyl-terminal (B-chain or light-chain) region contains a serine protease domain [17]. Unlike uPA, single-chain tPA has significant enzymatic activity [18]. The finger domain and kringle domain are important in binding of tPA to fibrin [19]. Endothelial cells in particular produce tPA [20, 21], as do human keratinocytes, melanocytes and neurons [22–24]. Of neoplastic cells, tPA can be produced by melanoma, neuroblastoma, ovarian cancer, breast cancer and pancreatic cancer cells [25–28].

uPA

The urokinase mRNA of 2.5 kb [29] encodes a single-chain glycoprotein with a molecular mass of 53 kDa [30–32]. The enzyme is activated by cleavage of a single peptide bond. Similarly to uPA, this cleavage can be catalyzed by plasmin, for example [32–34]. Active urokinase is a two-chain form held together by a single disulfide bond. The tertiary structure of uPA is composed of three different domains. The N-terminal A-chain (light chain) includes a growth factor domain followed by one kringle domain [35, 36]. These together are known as the amino-terminal fragment (ATF) (1–135 amino acids) [37]. The receptor-binding site has been mapped to the growth factor domain of the ATF fragment [38]. In the carboxyl-terminal region, also known as B-chain (heavy chain), uPA has a serine protease domain. The two chains are linked with a connecting peptide (tPA does not have it). uPA is secreted by many types of cancer cells, including breast, colon, ovary, gastric, cervix, endometrium, bladder, kidney and brain tumor tissues, in higher amounts than by the corresponding normal tissues [39]. While the cells producing uPA in many cancer tissues remain unidentified [40], in colon carcinoma urokinase has been established to be secreted mainly by the cancer cells themselves [41].

Receptors for plasminogen activators

Cell membrane-bound high-affinity receptors for tPA have not been identified. Yet, tPA binds to many proteins, and fibrin is by far the most efficient binding protein. In at least one respect, fibrin can be said to act as a receptor for tPA; in the presence of fibrin, tPA-catalyzed plasminogen activation occurs at least 100-fold more effectively than in its absence [19, 42]. Even if not a true receptor, it is at least a major cofactor for tPA. Other tPA candidate receptors include mannose-6-phosphate/insulin-like growth factor

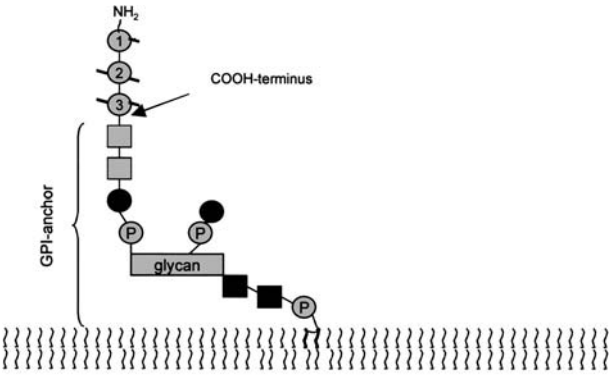

Figure 2. Schematic presentation of GPI-anchored uPAR. uPAR consists of three homolgous domains and it is bound via glycolsyl-phosphatidyl-inositol moiety (GPI anchor) to the cell surface. Domains 1 and 3 are responsible for uPA binding.

(GF) II receptor, annexin II and amphoterin [10, 43, 44]. In addition, tPA binds to extracellular matrix components such as laminin and fibronectin [45, 46].

uPA has high-affinity binding sites on the cell surface [37, 47, 48]. Urokinase plasminogen activator receptor (CD87/uPAR) (fig. 2), has a molecular mass of ~55 kDa [49]. The mRNA for uPAR is 1.4 kb [50]. Binding of uPA to its receptor is species-specific, i.e. human uPA does not bind to murine uPAR and murine uPA does not bind to human uPAR [51]. Fully processed uPAR is heavily glycosylated [52]. In its carboxyl-terminal region, uPAR has a glycosyl-phosphatidyl-inositol moiety (GPI anchor) by which uPAR is integrated into the outer leaflet of the cell membrane [53]. The tertiary structure of uPAR is formed by three homologous repeats known as domains 1, 2 and 3 (D1, D2, D3) [54, 55]. Based on its protein structure, uPAR belongs to the glycolipid-anchored Ly-6 superfamily of proteins [56]. The ligand-binding site is located in D1, which is also the first repeat starting from the amino-terminus [54]. Purified or cell membrane-bound uPAR is cleaved either partially or totally [53, 54, 57], and it therefore exists in soluble form. High levels of soluble uPAR (suPAR) can be measured, for example, in patients with acute myeloid leukemia [58]. uPAR is expressed at elevated levels in breast colon, gastric and certain lung carcinoma tissues [39]. Disagreement exists between different laboratories as to which cells produce uPAR, whether it is produced by stromal cells such as macrophages or by cancer cells in breast and colon carcinoma tissues [59]. In ductal breast carcinomas uPAR is associated with stromal cells [40].

PAIs

PAI-1 and PAI-2

The two known physiological inhibitors for uPA and tPA are plasminogen activator inhibitors-1 and -2 (PAI-1 and PAI-2, respectively). PAI-1 and PAI-2 belong to the gene family of serine protease inhibitors called serpins [60–62]. Both are glycoproteins. The molecular mass of PAI-1 is ~54 kDa [63]. PAI-1 has two mRNAs (2.4 and 3.2 kb), which is thought to be due to alternative polyadenylation sites. Serpins can adopt a variety of conformations under physiological conditions [64]. PAI-1 can exist in its native inhibitory form, in an inactive latent form, in complexes with proteinases and in a cleaved substrate form [65, 66]. In addition, PAI-1 can form complexes with vitronectin, while simultaneously retaining its activity [67, 68]. Endothelial cells as well as many other types of cells in culture secrete PAI-1 [69–71]. Platelets contain a large pool of PAI-1, mostly in an inactive form [71].

PAI-1 forms complexes both with single-chain and two-chain tPA and with two-chain uPA [72]. However, PAI-1 has also been shown to form a reversible complex with single-chain uPA [73]. Receptor-bound active uPA is inhibited by PAI-1 and PAI-2 [74–76], which leads to the inhibition of plasminogen activation.

Based on the amino acid and gene structure, PAI-2 belongs to a subgroup of the serpin superfamily, ovalbumin-type serpins [62, 77]. Its gene is transcribed to a 2.0-kb mRNA [62]. PAI-2 protein exists in two forms: a nonglycosylated intracellular form and a glycosylated, secreted extracellular form [78]. PAI-2 can also be detected intracellularly as a polymer [79]. The molecular mass of the intracellular form is ~42 kDa and that of the secreted form 60 kDa [80]. PAI-2 inhibits uPA rapidly, but tPA very slowly [71]. PAI-2 is expressed in placental trophoblasts [81], as an inflammatory response in macrophages and also in keratinocytes under certain conditions [22, 78].

α_2-antiplasmin and α_2-macroglobulin

The serpin inhibitor α_2-antiplasmin is the primary inhibitor of plasmin [82]. It forms a complex with plasmin by occupying the plasmin kringle domains, i.e. the lysine-binding sites. It is a fast inhibitor of free plasmin both in circulation and in solution. When plasmin/plasminogen is bound to the cell surface, its lysine binding sites are occupied, and therefore α_2-antiplasmin acts more slowly [6]. α_2-Macroglobulin is found in circulation in vast quantities [83]. It is a large glycoprotein which can trap proteinases and their inhibitor complexes. α_2-Macroglobulin complexes are internalized by means of low-density lipoprotein-related receptor LRP [84]. α_2-Macroglobulin binds plasminogen activators and their inhibitor complexes as well as plasmin. Cell-bound plasmin is protected from α_2-macroglobulin [85].

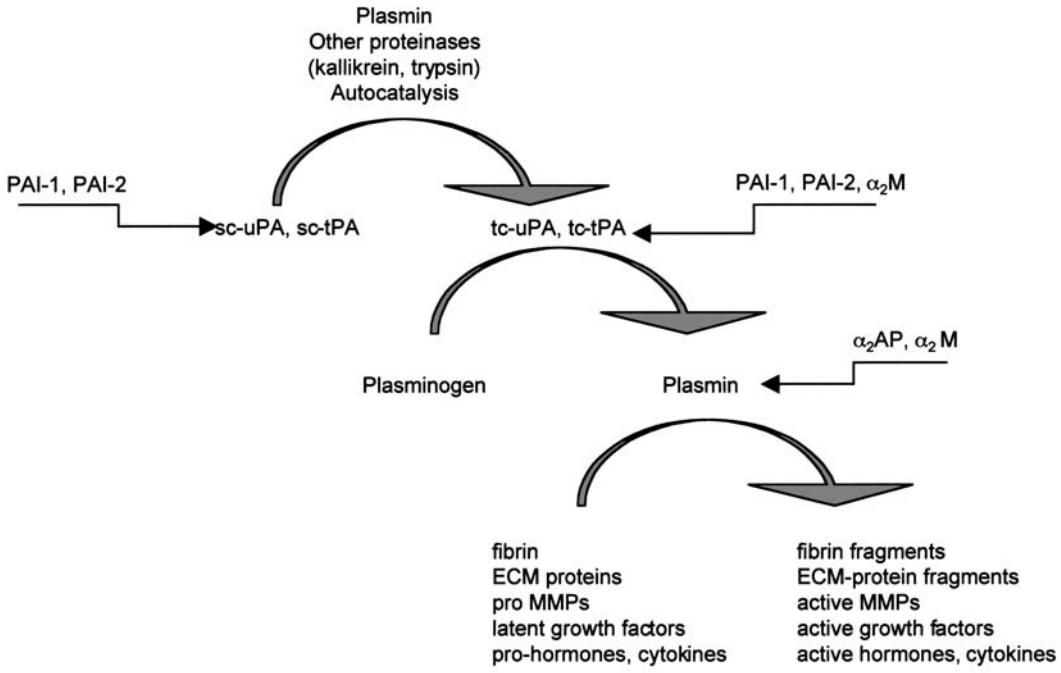

Figure 3. Plasminogen activation cascade. α_2M, alpha-2-macroglobulin; α_2AP, alpha-2-antiplasmin.

Physiological and pathophysiological roles of plasminogen activation

Consequences of plasminogen activation (see fig. 3)

The discovery that plasmin solubilizes the fibrin network led to the utilization of plasminogen activators as thrombolytic agents in cardiovascular diseases and ischemic stroke [19, 86]. In knockout mice deficiency of plasminogen and its activators has been demonstrated to cause severe thrombosis [87, 88]. uPAR-uPA participates in fibrinolysis, but extravascularly [89]. Plasmin is necessary in wound healing [90, 91], and matrix metalloproteinases (MMPs) are needed to complete the wound healing process [92]. In humans, plasminogen deficiency is a common cause of ligneous conjunctivitis, a disease characterized by pseudomembranous lesions mainly in tarsal conjunctivae [93]. In these patients no severe thrombosis has been reported [93]. MMPs have the capacity to split fibrin in the neovascularization process [94]. Do MMPs provide compensating fibrinolysis in vasculature?

In many physiological and pathophysiological conditions, plasminogen activators and MMPs act in concert. Proteolytic processing of the structural hemidesmosome protein laminin-5 is an example of one such target protein. Plasmin and tPA regulate the proteolytic processing of the α_3-chain of laminin-5, which results in the assembly of stabilized hemidesmosome structures and cessation of cell migration [95]. The same group demonstrated that uPA, plasmin and integrin ligation with uPAR was involved in the processing of the laminin-5 α_3-chain, thus reducing cell motility [96]. Cleavage of the laminin-5 γ_2-chain by MMP-2 induces cell migration [97], although MT1-MMP was later shown to be mainly responsible for the truncated form of the laminin-5 γ_2-chain with or without MMP-2 [98]. Interestingly, uPAR and the truncated form of the laminin-5 γ_2-chain are coexpressed in colon adenocarcinomas at the front of invasive cancer cell islands [99]. Moreover, the overexpression of uPA and uPAR (bitransgenic mouse) in basal mouse keratinocytes increased plasminogen activation and cooperated in pathogenic proteolysis, which led to partial disruption of hemidesmosomes and epidermal blistering [100]. In the same study, MMP-9 and MMP-2 were shown to be activated only in bitransgenic mice. The proteolytic conversion of plasminogen to plasmin can occur without uPAR [101]. Plasmin can activate several MMPs in vivo; for example, during aneurysm formation MMP-3, -9, -12, and -13 are activated [102].

uPA-uPAR complex formation on the cell surface has proteolytic, cell migratory, adhesive and more recently characterized chemotactic effects. The cleavage of uPAR between D1 and D2 exposes an epitope fragment on the side of D2D3 uPAR which is chemotactic [103]. The binding of uPA to uPAR seems to be needed to unmask the chemotactic uPAR sequence. Whether the cleavage occurs by uPA or other proteinases in vivo remains to be seen [103, 104]. It is not clear whether the D2D3 soluble form, the membrane-attached form or both induce the chemotactic effect in vivo [105]. The D2D3 sequence of uPAR interacts with the chemotactic formyl peptide receptor (FPR)/FPR-like receptor-1 (FPRL1) [106]. However, in arteriogenesis, uPA-mediated infiltration of leu-

kocytes is not dependent on uPAR [107]. The presence of the two activators in the joints of rheumatoid arthritis patients seems to have opposing effects on disease severity: tPA has a protective role and uPA is deleterious [108, 109]. This is possibly based on tPA-mediated fibrin clearance and uPA-mediated recruitment of inflammatory cells and cytokines in arthritic joints.

Plasmin has the capacity to release and activate growth factors, hormones and proteases such as transforming growth factor-β (TGF-β) [110, 111], fibroblast growth factor (FGF) [112], insulin-like growth factor-binding protein-4 (IGFBP-4) [113] and, as mentioned above, latent collagenases [114, 115]. In vitro, plasmin converts proinsulin, adrenocorticotrophin (ACTH) [116] and interleukin-1β (IL-1β) [117] to their active forms. In addition, uPA has been shown to activate hepatocyte growth factor (HGF) in vitro [118]. Plasmin can also activate latent TGF-β in a reaction where uPAR facilitates this activation on the cell surface via bound uPA [119].

Plasminogen activation and tumorigenesis

The connection between increased fibrinolytic activity and cancer was initially described more than 80 years ago. The pioneers Alex Carrel and Albert Fischer, among others, discovered that cancer cells continuously dissolve plasma clots in culture by proteolytic digestion [120]. In the 1970s, the evidence was strengthened by plasminogen activators being produced in higher amounts in transformed cell lines [121]. Cancer cells have an enormous capacity to migrate. Plasmin, with its degrading capacity, has been thought to facilitate tumor cell passage through barriers such as basement membranes and interstitial connective tissue, and migration to distant body sites. Furthermore, plasmin's ability to active growth factors and latent proteinases has been thought to contribute to cancer cell invasion. The invasion of cancer cells is associated with the secretion of high amounts of uPA, and it predicts aggressive disease [122]. This has also been shown in reverse. Human epidermoid carcinoma cells lose their malignant behavior if they are cultured for a prolonged time. This is accompanied by a number of changes, including reduced uPA synthesis [123]. High levels of uPA and uPAR are related to poor prognosis, and it therefore came as a surprise that high PAI-1 levels are also a marker for poor prognosis in certain cancers [124]. Recently, it has become clear that the situation is much more complex and that alternative routes exist within the plasminogen activation system. The components of the plasminogen activation cascade, namely uPA, uPAR and PAI-1, take part in cell adhesion and migration. This function is partly independent of their proteolytic or inhibitory functions (see below section on interaction of plasminogen activation components with adhesion molecules). The role of PAI-1 in cancer cells is unclear. PAI-1 can disturb the interaction between vitronectin and its receptor [125]. PAI-1 might be also related to angiogenesis in which it seems to be involved. Alternatively, it may be due to either the protease-inhibitory or the adhesive function of PAI-1 or both [126, 127]. Both the protease-inhibitory and the adhesive function via vitronectin/integrin participate in the regulation of cancer metastasis [128].

Role of tPA in the brain

In 1981, tPA was demonstrated to be released at the neuronal growth cone [129]. Morphological differentiation of neuroblastoma cells was accompanied by tPA induction, which also suggested it to have a role in neuronal cell functions [26]. Involvement of plasmin(ogen) as well as tPA in brain function has been studied in knockout mice. tPA participates in neuronal plasticity such as involvement in memory and learning activities [130]. Moreover, tPA has been shown to operate in stress-induced neuronal plasticity [131]. Corticosteroids have a role in memory and learning processes [132], and they are known as stress hormones. Mice deficient in tPA have higher stress tolerance and decreased neuronal remodeling within the amygdala. In addition, tPA-deficient mice show elevated and extended strength of corticosteroid levels after restraint stress [131]. Regulation of hippocampal synaptic reorganization is facilitated by tPA both in a plasmin-dependent and -independent manner [133]. A proteoglycan is one target of plasmin-mediated cleavage in the central nervous system [134]. tPA can promote mossy fiber extension without its catalytic activity [134]. On the other hand, tPA can also cause neuronal cell death [135]. Plasmin is involved in hippocampal neuronal cell death and in the destruction of laminin via activation by tPA [136].

The N-methyl-D-aspartate receptors (NMDARs) are classical memory and learning receptors. The NMDARs are glutamate-gated ion channels. Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system [137, 138]. Activated presynaptic terminals release glutamate, which binds to NMDAR in the plasma membrane of depolarized postsynaptic cells. Glutamate opens the NMDAR channel, allowing Ca^{2+} (or Na^{+}, K^{+}) to enter the cell. Elevated Ca^{2+} acts as an intracellular mediator, e.g. by activating enzymes. Excess of glutamate causes Ca^{2+} to enter cells and can result in neurotoxicity. When severe, it leads to cell necrosis and in a milder form to cell apoptosis. Glutamate and its receptors play a critical role in pathological conditions such as cerebral ischemia, traumatic brain injury, and acute and chronic neurodegeneration.

Patients with ischemic stroke are treated with tPA to induce fibrinolysis. However, this treatment can cause neuronal cell damage; the underlying mechanism remains

obscure. It has been demonstrated that tPA can augment neuronal cell destruction by directly cleaving the NMDA receptor [139]. tPA-cleaved truncated NMDAR forms are thought to allow excessive Ca^{2+} influx, leading to neurotoxicity and neuronal damage [140]. Inactive tPA has a beneficial role in brain injury; without its proteolytic activity, it can activate microglial cells, which then further enhance their activation by releasing tPA [141]. The non-proteolytic action of tPA does not fit with the well-known fact that it has a high intrinsic activity in zymogen form, and it efficiently catalyzes plasminogen activation in the presence of fibrin or other binding proteins.

PAI-1 and PAI-2 in diseases

Disturbances in the amount of PAI-1 in plasma and its structural defects have been related to pathological conditions such as cardiovascular and thromboembolic disease [142] and bleeding disorders [143]. The level of plasma PAI-1 is elevated in atherosclerosis and it may contribute to its development [144]. PAI-1 is elevated in diabetes or in the evolutionarily related disease insulin resistance/insulin resistance syndrome, which both predispose to cardiovascular disease [145–147]. Hypofibrinolytic conditions are characterized with pathological matrix accumulation diseases such as renal fibrosis, where elevated PAI-1 levels can attenuate matrix turnover [148]. PAI-1 is also an acute-phase reactant and has been shown to be induced in mouse hepatic cells in response to tissue injury [149]. Regardless of its linkage to many severe diseases, the origin of plasma PAI-1 remains unknown.

In contrast to PAI-1, elevated levels of PAI-2 predict a favorable prognosis in primary breast cancer [39, 124]. The unusual intracellular occurrence of PAI-2 has long been a mystery. It has been proposed that cytosolic PAI-2 participates in host defense against viral infection. PAI-2 is also required for cell survival, since it inhibits tumor necorosis factor-α (TNF-α)-induced apoptosis [150]. However, in a study where PAI-2 protected cells from certain viral cytopathic effects, it was discovered that PAI-2 mediated interferon production, which led to expression of antiviral genes [151]. PAI-2 also has an unidentified role in the regulation of carcinogenesis caused by exposure to chemical carcinogens. High expression of PAI-2 mRNA is induced rapidly by dioxin in human keratinocytes [152]. The overexpression of PAI-2 in transgenic mice had an enhancing effect on papilloma formation, some of which progressed to carcinoma, as studied by the multistage skin carcinogenesis model [153]. Moreover, as the lesions progressed to invasive carcinoma, a change occurred in gene expression; PAI-2 was turned off and uPA turned on [153]. Massive apoptosis in papillomas of control mice developed after the cessation of phorbol 12-myristate 13-acetate (PMA) application, while in transgenic mice limited apoptosis occurred. This might be related to the recently reported PAI-2 expression in the progression of squamous cell carcinoma. PAI-2 is expressed in high levels in normal and dysplastic keratinocytes, whereas its expression is considerably decreased in squamous cell carcinoma [154].

Regulation of plasminogen activation

A key question in the plasminogen activation field has been how the entire activation cascade is initiated. All enzymes in the PA system are secreted in proforms (plasminogen, uPA and tPA). Single-chain (proenzyme) tPA has significantly higher intrinsic enzymatic activity than uPA [155, 156]. In fact, there has been debate about whether uPA has any intrinsic enzymatic activity in its proenzyme form [33, 157]. Some reports also describe the proenzyme form of uPA as having intrinsic enzymatic activity [158]. Some studies suggest that PAI-1 cannot bind and inhibit pro-urokinase [72], and others claim that it can form reversible enzyme-inhibitor complexes [73]. PAI-1 has, however, been shown to behave as a substrate to its enzyme. After forming a complex (uPA-PAI-1), uPA is released in active form and PAI-1 in inactive form [65]. Activation of pro-urokinase or plasminogen can occur by kallikrein [159, 160]. In addition, plasminogen, which circulates in closed conformation, will adopt an open conformation upon binding on fibrin [161, 162]. In this open conformation, it is believed to be more readily activated.

Endocytosis

Endocytosis regulates the clearance of uPA and tPA from the cell surface, the circulation and the extracellular space. Receptor-bound urokinase is most efficiently internalized in complex with its inhibitor, PAI-1 [163]. This triad is endocytosed by means of the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α_2M/LRP, same as LRP) or very low-density lipoprotein protein (VLDL) from the cell surface [164–168]. Internalized uPAR is recycled to the cell surface [169]. The clearance of tPA-PAI (as well as of uPA-PAI-1) complexes from the circulation occurs via LRP in hepatic cells [167]. uPAR is needed to present the uPA-PAI complex to α_2M/LRP [170]. Such a receptor is not known for tPA.

Effects of hormones, growth factors and cytokines on plasminogen activation (see table 1)

A retinoic acid receptor response element is located in the human tPA gene [171]. Retinoic acid induces tPA expression in microvascular endothelial, oral squamous carcinoma and neuroblastoma cells [172, 173]. More recently,

Table 1. Effect of growth factors, cytokines and hormones on the plasminogen activation system.

Effector	Cells	tPA	uPA	uPAR	PAI-1
RA	neuroblastoma	increase	no effect	no effect	no effect
	oral squamous carcinoma cells	increase	no effect		
	microvascular endothelial cells	increase	increase		
EGF	epidermoid carcinoma (A431)	increase	increase		no effect
	colon carcinoma cells		decrease	increase	
	neoplastic uroepithelium cells	no effect	minor decrease		
	normal uroepithelium cells	increase	increase		
	squamous cell carcinoma (UM-SCC-1)	no effect	increase	increase	
HGF/SF	squamous cell carcinoma (UM-SCC-1)	no effect	increase	increase	
TGF-β	lung carcinoma (A549)	no effect	increase	increase	
	retinal pigment epithelium (RPE) cells			increase	
	breast cancer cells	decrease	increase	increase	
VEGF	endothelia cells (serum needed)	increase			increase
bFGF	endothelial cells (serum needed)	increase			increase
bFGF, FGF-2	pancreatic carcinoma	increase			decrease
FGF-7	prostatic epithelial cells		increase		
IL-4	vascular SMC (serum needed)	increase	no effect		decrease
IFN-γ	vascular SMC	decrease	no effect		
	monocytes			increase	
	colon carcinoma cells (HTC116)			increase	
IFN-α	colon carcinoma cells (HTC116)			increase	
PDGF	vascular SMC	increase	no effect		
IL-1	keratinocytes	increase			

a multihormone-responsive enhancer element, which is activated by glucocorticoids, progesterone, androgens and mineralocorticoids [174], was identified in the tPA promoter [174], and it has four glucocorticoid receptor binding sites. The human uPA gene enhancer region contains PEA3 (polyoma enhancer A-binding protein)/AP-1A (activating protein-1) and AP-1B sites which are pivotal for the induction of uPA gene transcription by PMA and epidermal growth factor (EGF) [175, 176]. The PEA3/AP-1 transcription factors have been shown to bind in a cell type-specific manner on the uPA gene enhancer element [177]. The human tPA gene contains AP-1 sites, too [178]. uPA gene transcription is regulated by the family of nuclear factor-kappa B (NF-κB) transcription factor proteins [179–181]. uPA gene transcription repression is regulated by heterodimeric AP-1 transcription factors [182].

PAI-1 has a glucocorticoid response element (GRE) site in its gene-regulating elements [183]. An insulin-response element binding Sp 1 transcription factor has been mapped to the PAI-1 gene promoter region [184]. In hyperglycemic conditions overproduction of superoxide and activation of the hexosamine pathway increases Sp1 glycosylation, which in turn increases PAI-1 and TGF-β promoter activity [185]. Elevated PAI-1 in obese mice is regulated by TNF-α [186].

The response of uPAR gene expression to TGF-β varies strongly in different cell lines. This was studied in several human normal and neoplastic cell lines [187]. For example, in lung carcinoma cells, TGF-β enhanced uPAR mRNA. In addition, the secretion of uPA and PAI-1, but not of tPA, was affected by TGF-β [187]. In human retinal pigment epithelial (RPE) cells TGF-β induced rapid and transient uPAR expression [188].

TGF-β is a well-known stimulator of PAI-1 gene transcription [189, 190]. The TGF-β-inducible transcription factors SMAD3 and SMAD4 also bind to PAI-1 TGF-β response elements in the PAI-1 gene promoter [191]. TGF-β enhancement of PAI-1 expression can be repressed by glucocorticoids. This occurs with protein-protein interaction between GRE and TGF-β response elements binding the proteins SMAD3 and SMAD4 [192]. TGF-β regulates the invasiveness of breast cancer cells by enhancing secretion of uPA, resulting in stimulation of plasmin formation. The amounts of uPAR and PAI-1 are also increased. tPA levels, by contrast, are decreased with TGF-β [193].

Addition of EGF to human epidermoid carcinoma (A431) cells was shown to downregulate EGF receptors (EGFRs) with a concomitant increase in plasminogen activators [194]. Furthermore, the EGF-induced tPA activity has been shown to be hidden by PAI-1 in the A431 cell line [195]. In a colon carcinoma cell line, EGF decreased uPA production preceding the uPAR increase. Insulin and transferrin had no effect on uPA and uPAR expression [196]. In human squamous cell carcinoma cells, addition of EGF and scatter factor (SF, HGF) had an inducing ef-

fect both on uPA and uPAR [197]. Nevertheless, MMPs induced invasion of these cells. Normal human uroepithelial cells produce tPA and uPA but neoplastic epithelia uPA alone. EGF had a slight effect on uPA expression in neoplastic uroepithelial cells. tPA production, by contrast, diminished after the cells reached saturation. Addition of EGF had a considerable increasing effect on tPA expression in normal epithelium [198].

Neither vascular endothelial growth factor (VEGF) nor basic fibroblastic growth factor (bFGF) alone affected tPA levels, but together they induced tPA in bovine endothelial cells. Other serum factors seem to be needed because the VEGF- and bFGF-induced tPA levels were not as high as in the presence of serum [199]. VEGF-B led to increased expression of uPA as well as of PAI-1 [200]. In mouse smooth muscle cells, the mitogenic or proliferative response by platelet-derived growth factor (PDGF) or bFGF was reduced in mice deficient of tPA or uPA. The effect of PDGF was shown to require the presence of tPA, and bFGF the presence of uPA. Both plasminogen activator mRNAs were induced severalfold, uPA with bFGF and tPA with PDGF [201]. In pancreatic carcinoma cells, FGF-2 regulated tPA and PAI-1 expression [202]. In the prostate epithelial cell line, FGF-7 induced invasion of cells accompanied by overexpression of MMP-1 and uPA [203].

Among the cytokines, IL-1β increased tPA production by a human keratinocytes cell line [204]. In human aortic smooth muscle cells, IL-4 increased tPA antigen and interferon-γ (IFN-γ) decreased the effect of IL-4 in the presence of serum. The presence of PDGF was also shown to have an effect on tPA antigen levels and thereby on muscle cell migration [205]. The fibrinolytic potential of human endothelial cells was stimulated by lipopolysaccharide (LPS), TNF-α and IFN-γ [206]. Monocytes, which take part in inflammatory reactions, apparently respond to IFN-γ and TNF-α by increasing uPA binding on the cell surface; when combined, the two factors are strongly synergistic [207]. IFN-α or -γ upregulates uPAR protein expression in the colon cancer cell line [208]. Plasminogen is regulated by IL-6 [209]. Furthermore, an IL-6 response element has been mapped to the murine plasminogen promoter sequence [210]. In addition, a potential glucocorticoid-response element is present in the plasminogen gene [209].

Glucocorticoids have a strong impact on plasminogen activation (see table 2)

The repressing and increasing effects of glucocorticoids are due to the relative impact of these steroids on different components of the PA system. They repress plasminogen activation either by increasing inhibitor (mainly

Table 2. Effect of hydrocortisone or dexamethasone on plasminogen activation.

Cells	PA-activity	tPA	uPA	PAI-1	PAI-2
Fibrosarcoma HT-1080 cell line	decrease	increase	decrease	increase	decrease
Melanoma cell lines:					
Malme-3M	no effect	no effect			
RPMI 8252	no effect	no effect			
SK-MEL-2	no effect	no effect			
SK-MEL-26	no effect	no effect			
SK-MEL-27	no effect	no effect			
MeWo	no effect	no effect			
Embryonic lung cells	inhibit				
Embryonal kidney cells (HEK)	inhibit		decrease		
Uveal melanocytes	inhibit		decrease		
Renal carcinoma (Caki-1)	inhibit		decrease		
Renal carcinoma (Caki-2)	inhibit				
Lung adenocarcinoma (Calu-3)	inhibit				
Mammary carcinoma cells (MDA-MB-231)		decrease	decrease	increase	
Rat hepatoma cells	decrease	no effect		increase	
Ovarian carcinoma (OVCA 433)	decrease	decrease	decrease		
Rat ovarian granulosa cells		increase			
Murine keratinocytes	decrease	no effect	no effect	increase	
Mammary gland involution	inhibit	decrease	decrease		
Rat prostate gland involution	inhibit	decrease	decrease	no effect	
Neoplastic prostate organ culture		decrease	decrease		
Benign prostate organ culture		decrease	decrease		
Rat organ tongue culture	inhibit	decrease			
Patients with cirrhosis, plasma levels	decrease				
Human cervical epithelial cells (HCE16/3)		decrease	decrease	no effect	

PAI-1) synthesis [211] or by decreasing activator synthesis [212, 213]. In human HT-1080 fibrosarcoma cells, dexamethasone was demonstrated to increase tPA and PAI-1 synthesis and decrease uPA and PAI-2 synthesis, the net result being decreased PA activity in the cell culture medium [214]. In addition, in HT-1080 cells, dexamethasone reduced the number of plasmin-binding sites on the cell surface [215].

Glucocorticoids can also have the opposite effect on the synthesis of the plasminogen two activators [216] without affecting inhibitors in the same cells. In human embryonic or tumor-derived carcinoma cell cultures, dexamethasone decreased plasminogen activator activity but had no repressive effect on six melanoma-derived cell cultures, which mainly produced tPA [217]. In fact, dexamethasone increased tPA activity in these cultures. In rat hepatic cells dexamethasone increased tPA gene transcription, and when combined with cyclic AMP (cAMP), tPA was upregulated further [218]. The increase in tPA activity by dexamethasone was also seen in rat granulosa cells under the influence of certain hormones and growth factors [219]. However, in human ovarian carcinoma cells, dexamethasone decreased total PA activity by 95% [27]. The decrease in uPA activity preceded the effect on tPA activity. Moreover, there was a marked effect on cell morphology by dexamethasone. In human embryonic lung cells, glucocorticoids have a suppressive effect on PA synthesis [220].

One example of the impact of hydrocortisone on plasminogen activator(s) is given by tissue remodeling events such as mammary gland involution. Hydrocortisone inhibits mammary gland involution [221, 222]. uPA, as well as MMPs, are strongly upregulated in mammary gland involution. In hydrocortisone-treated mice, the involution regresses, and the proteases (including uPA) are strongly repressed [223]. Another example of tissue remodeling is provided by castrated rats. Prostate involution was accompanied by uPA and tPA upregulation, which was retarded by the administration of hydrocortisone, followed by downregulation of the uPA and tPA activities (especially the 30-kDa form of uPA) [224]. Benign and malignant prostate tissues were shown to contain both types of plasminogen activators, albeit more tPA than uPA. The presence of hydrocortisone in prostate tissue organ culture led to a marked decrease of plasminogen activators [225].

Early reports demonstrated that administration of corticotrophin significantly decreases the elevated fibrinolysis seen in patients with liver cirrhosis [226]. In rat tongue organ culture, the squamous tongue epithelium was shown to produce tPA. The addition of hydrocortisone to the culture medium led to a marked repression of tPA activity [227]. In the same study, hydrocortisone-treated cultures were observed to have better tissue integrity, although this could not been confirmed to be the result of the decrease in tPA. Maintenance of mammary epithelial cell differentiation is partly due to extracellular matrix turnover caused by the inhibitory effect of glucocorticoids [228].

Keratinocyte growth factor (KGF) programs HPV-16-immortalized human cervical epithelial cells (HCE16/3) to a more advanced malignant phenotype [229]. KGF increases uPA, with little or no tPA in these cells [230]. tPA is under the strong repressive control of hydrocortisone in the HCE16/3 cell culture system [231]. The removal of hydrocortisone altered cell morphology of HCE16/3 cells, which was accompanied with enhanced tPA activity and mRNA expression. Interestingly, deficiency of tPA is shown to block kidney tubular epithelial mesenchymal transition (EMT) [232]. In tPA-deficient mice MMP-9 gene expression was markedly reduced, which reflected better basement membrane integrity [232]. tPA regulates MMP-9 expression in human brain endothelial cells [233]. tPA can have a yet unidentified role in epithelial cell physiology as well as in pathophysiology. Hydrocortisone has also been studied in murine keratinocytes, where it was found to have an increasing effect on PAI-1 mRNA and antigen levels, but not on tPA and uPA mRNA [211]. In this system, plasminogen activation was mainly suppressed due to the increased PAI-1 production.

Both PAI-1 and tPA plasma levels display a circadian rhythm for unknown reasons. The tPA activity is lowest early in the morning and increases during the day, while PAI-1 activity is highest in the morning [142]. In addition, in the rare disease Cushing‘s syndrome (hypercortisolemia) fibrinolysis is impaired possibly due to high PAI-1 levels [234]. Both tPA and PAI-1 are regulated by glucocorticoids and contain GRE in their genes. Thus, it is worth noting that cortisol also displays a circadian rhythm. In human plasma, cortisol is at its maximum level early in the morning, declining throughout the day [235].

Interaction of plasminogen activation components with adhesion molecules (see table 3 and fig. 4)

In vitro, urokinase is localized on the cell surface at focal contacts tips of microspikes, cell-cell contacts and lamellipodia [236, 237]. uPAR is GPI-anchored [53], which prompted researchers to search for associated molecules. Finding an explanation for those uPA-uPAR-related cellular events that were independent of uPA’s proteolytic activity (e.g. cell migration caused by the formation of uPA-uPAR complexes) was of particular interest [238–240].

Earlier findings of PAI-1 being deposited on the substratum of cell cultures [236] and the formation of complexes with vitronectin while retaining its activity [67] suggested that PAI-1 is more than an inhibitor. The first

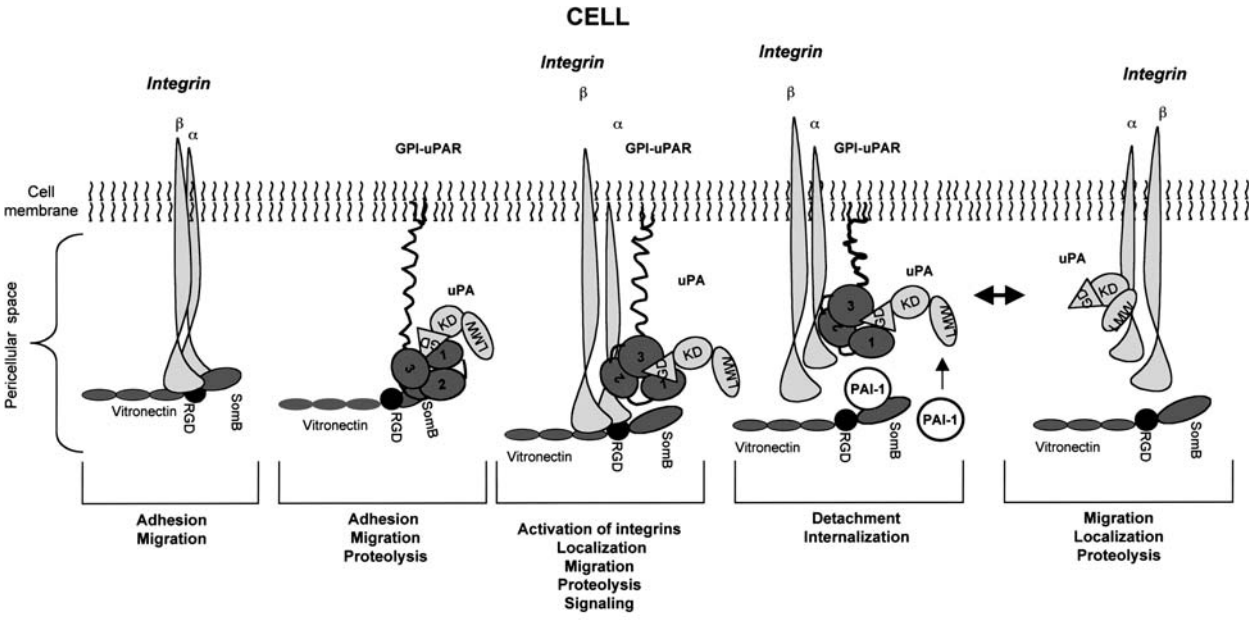

Figure 4. Schematic model for uPAR, uPA, PAI-1 interactions with some pericellular adhesion molecules. Integrins binds to the RGD sequence on ligands. uPAR binds the growth factor domain (GD) of uPA. In addition, uPAR binds to the integrins as well as somatomedin-B domain (SomB) of vitronectin. uPA seems to be needed for optimal uPAR binding either to integrins or their ligands. Binding of uPA to uPAR promotes cell adhesion and migation. Both uPAR and PAI-1 recognize the SomB domain of vitronectin. PAI-1, if given or expressed in excess, can inhibit both uPAR-vitronectin and integrin-vitronectin interactions. The PAI-1 binding site on vitronectin is close to the RGD sequence, and PAI-1 has higher affinity towards vitronectin than uPAR. uPA has the capacity to bind both receptors (uPAR and integrins) simultaneously, but is also capable of binding to integrin independently of uPAR. The protease domain (LMW, low molecular weight) and kringle domain (KD) of uPA recognize integrins. In addition, uPA bound to integrins is capable of impairing recognition ligands of integrin (other than uPAR).

Table 3. Plasminogen activation components and adhesion molecules. VN, vitronectin; FN, fibronectin.

PA Components	Interacting molecules	Adhesive/deadhesive functions
uPAR	VN	adhesion
uPAR+PAI-1	VN	detachment
uPAR+uPA	VN, FN, $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_1$	adhesion
uPAR+uPA +PAI-1	VN, $\alpha_v\beta_3$, $\alpha_v\beta_5$	detachment
uPAR+uPA +PAI-1	VN, $\alpha_v\beta_3$, $\alpha_v\beta_5$, LRP	detachment, integrin inactivation, endocytosis
uPAR (uPA?)	$\alpha_4\beta_1$, $\alpha_v\beta_3$, $\alpha_9\beta_1$	cell-cell adhesion
uPA	uPAR, $L_m\beta_2$	adhesion

demonstrations showed that the addition of PAI-1 antibodies disturbs cell adhesion [241]. This was shown to be due to the disruption of vitronectin-dependent adhesion [242]. Another group also identified uPA and uPAR to be involved in cell adhesion. The adhesion of human myeloid cells, provoked by PMA, could be abolished by uPA antibodies and be reversed by the addition of the ATF fragment of uPA [243]. Furthermore, PAI-1 was suggested to be responsible for the reduced adherence, but the reason was presumed to be increased PAI-1-uPA turnover [244]. While the adhesion was subsequently verified to be vitronectin-uPA dependent, identification of the vitronectin-binding sites (other than integrins) on the cell surface failed [245]. However, in the same year vitronectin was reported to bind with high affinity to uPAR on cell the surface [246], which was further strengthened by concurrent uPA binding to its receptor. Vitronectin-uPAR binding was enhanced by uPA and inhibited by PAI-1 in human endothelial cells [247].

The distribution of uPA reflects the uPAR distribution on cell membranes [237]. It has been confirmed that uPAR is also found at the focal contacts, tips of microspikes, lamellipodia, cell-cell contacts of human rhabdomyosarcoma and embryonic fibroblasts, and it colocalized with $\alpha_v\beta_3$ integrins [248]. uPA was found to be one (perhaps not the only) candidate participating in the driving or anchoring of uPAR to focal contacts on the cell membrane [248].

Before long it was shown that uPAR is in close contact with integrins, as it could be specifically co-capped with CR3 (CD 11b/18, β_2 integrins) on neutrophil membranes [249] and was co-immunoprecipitated with β_2 integrin antibodies from monocyte lysates [250]. A few years later, the interaction between uPAR and integrins was confirmed in another study (this particular work focused

on β_1 integrins). In addition, it was proved that uPAR modifies integrin function and is found in caveolin-integrin complexes on the cell membranes [251]. In the same year, uPAR and PAI-1 were reported to have binding sites on vitronectin (not identical but overlapping), and thus PAI-1, which has a higher affinity to vitronectin, could compete for uPAR binding to vitronectin [252]. tPA was also capable of reversing the inhibitory effect of PAI-1 on adhesion. The relative amounts of uPA and PAI-1 determine whether an adhesive or nonadhesive effects occurs; more PAI-1 favors cell detachment from the substratum, while more uPA supports cell adhesion [125].

This picture became even more complicated when another group showed that PAI-1 is capable of inhibiting the migration of smooth muscle cells. This inhibition is possible due to sterical hindrance [253], since the PAI-1 attachment site on vitronectin overlaps with the binding site of vitronectin on the $\alpha_v\beta_3$ integrin. This inhibition was confirmed by others [254]. It has been demonstrated that $\alpha_v\beta_5$ integrin was needed for cell migration via uPA-uPAR on vitronectin and not the $\alpha_v\beta_3$ integrin [255]. It has further been demonstrated that in HT-1080 cells, uPAR co-localizes with several integrins, including β_1 and β_3, and it assembles with α_v, α_3, α_5 or α_6; the assembly depends on the composition of the extracellular matrix [256]. Immobilized PAI-1 can mediate adhesion and spreading of human myogenic cells, and this could be abolished with antibodies against the integrin $\alpha_v\beta_3$ [257]. An earlier finding [244] proposed that uPA-PAI-1 might cause cell detachment by PAI-1-uPA turnover, but this theory received little attention at that time. In more recent investigations LRP and uPA-uPAR-PAI-1-integrin internalization via LRP has been demonstrated to be involved in cell-adhesive functions [258, 259]. PAI-1 detached cells by disrupting the uPAR-vitronectin or the integrin-vitronectin interaction. In both situations, the PAI-1-mediated cell detachment was a uPA-dependent mechanism. It was suggested that detachment of cells by PAI-1 depends on the engaged amount of uPA-uPAR-integrin complexes relative to the total amount of active integrins [258]. Now, another group demonstrated that uPA can also interact with an integrin ($\alpha_M\beta_2$) on human neutrophil membranes [260]. This might partly explain the earlier studies where uPA binding to uPAR strengthened adhesion/migration or reversed the complex (uPA-PAI-1) formation. PA1-1 does not regulate uPA/tPA proteolytic function but seems to control uPA's adhesive function.

uPAR had been thought to associate with integrins and perhaps modify their functions [261], but recently, uPAR has been reported to be a ligand for integrins. uPAR was shown to directly compete with other ligands in binding to integrins [262]. In earlier studies uPAR was seen organized focally under cell-cell adhesion sites [248]. Later studies by others showed that integrin binding can indeed mediate cell-cell interactions [262].

Concluding Remarks

The knockout mice studies were disappointing, as the effects of deficiency of plasminogen, activators, uPAR and inhibitors were all mild. In fact, it is not the low levels of synthesis of plasminogen activation components which generates problems. In diseases affecting millions of people worldwide, cardiovascular disease and cancer, plasminogen activation is disturbed because of increased levels of these components. Although studies about levels of PAI-1 which promote cell migration show discrepancies [263], it is clear that high levels of PAI-1 predict poor prognosis in cancer and also in some chronic disease such as atherosclerosis. While the functions of PAI-1 other than anti-proteolytic are not well understood, it is possible that they have been overestimated. Also, the role of monocyte-associated plasminogen activation in atherosclerosis is not well understood [264, 265]. In migrating cancer cells uPA is secreted in high amounts as is PAI-1, which is secreted in the active conformation. Active PAI-1 has a high affinity towards active urokinase. Under such conditions excess uPA is likely activated either autocatalytically or by other proteinases. It may be primarily PAI-1 which regulates the overactive proteolytic machinery. Integrins and their ligands are part of the nonproteolytic action, perhaps regulating the proteolytic effects. Cell migration could be an indirect effect of excess amounts of uPA and PAI-1. Although high levels of PAI-1 predict poor prognosis, it is not excluded that its major function is still to inhibit urokinase.

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