



## Review

## The plasminogen activator inhibitor “paradox” in cancer

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## ABSTRACT

Proteolysis in general and specifically the plasminogen activating system regulated by urokinase (uPA) its specific receptor, the GPI membrane anchored urokinase receptor (uPAR) and the specific plasminogen activator inhibitor 1 (PAI-1) plays a major role in tumorigenesis, tumor progression, tumor invasion and metastasis formation. This is exemplified by a body of published work showing a positive correlation between the expression of uPA or uPAR in several tumors and their malignancy. It is generally assumed that such a “pro-malignant” effect of the uPA–uPAR system is mediated by increased local proteolysis thus favoring tumor invasion, by a pro-angiogenic effect of this system and also by uPA–uPAR signaling towards the tumor thereby shifting the tumor phenotype to a more “malignant” one. However, when tumor patients are analyzed for long term survival, those with high levels of the inhibitor of the system, PAI-1 have a much worse prognosis than those with lower PAI-1 levels. This indicates that increased overall proteolysis alone cannot be made responsible for the adverse effects of the plasminogen activating system in tumors. Moreover, it becomes increasingly evident that components of the fibrinolytic system secreted by the tumor cells themselves are not solely responsible for a correlation between the plasminogen activating system and tumor malignancy; components of the plasminogen activating system secreted by stroma cells or cells of the immune system such as macrophages contribute also to the impact of fibrinolysis on malignancy.

This review summarizes the evidence for the role of plasminogen activator inhibitor-1 in mediating the malignant phenotype and possible mechanism thereby trying to explain the “PAI-1 paradox in cancer” on a molecular level.

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## 1. Introduction

The fibrinolytic system represented by the cellular receptor of urokinase, the urokinase receptor (uPAR), a GPI linked protein, urokinase (uPA) itself and its specific inhibitor the plasmino-

gen activator inhibitor 1 (PAI-1) is clearly linked to malignancies by influencing tumor initiation, proliferation, migration, invasion, metastasis formation and apoptosis [1–4]. The basic knowledge on the function of the uPA/uPAR system comes from the analysis of mouse mutants but the knock out of uPA or uPAR has apparently no phenotype [5,6]. Urokinase is a plasminogen activator which transforms the zymogen plasminogen into the active protease plasmin. Plasmin in turn can degrade the extracellular matrix or activate other proteins (e.g. pro-metalloproteases and pro-growth factors). In addition to focusing proteolytic activity to the leading edge of invading cells [7], uPAR is also known to function as specific signaling receptor. Since uPAR is GPI-anchored to the membrane, it can, however, activate signaling only by interacting with extracellular or trans-membrane proteins.

In human tumors, the excessive expression of uPA and uPAR is a marker for an unfavorable clinical outcome [8]. Moreover, in human tumor xenografts, blockage of uPAR interaction with modified inactive uPA derivatives, blocks growth and metastasis of tumors (rev. in [2]). It is, however, unclear whether such correlation between “fibrinolysis” and “malignancy” is due to proteolytic effects or to other mechanism mediated by the uPA–uPAR system.

**Abbreviations:** uPA, urokinase-type plasminogen activator; uPAR, urokinase plasminogen activator receptor (CD87); tPA, tissue-type plasminogen activator; PAI-1, SERPINE1, plasminogen activator inhibitor 1; LRP-1, low density lipoprotein receptor related protein 1 (CD91) alpha-2-macroglobulin receptor; VLDLR, very low density lipoprotein receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; bFGF, fibroblast growth factor 2 (basic); MMP, matrix metalloproteinases; CRP, C-reactive protein; CEA, carcinoembryonic antigen; ECM, extracellular matrix; MAP kinase, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; AKT, v-akt murine thymoma viral oncogene homolog RAC serine/threonine-protein kinase protein kinase B; JAK, Janus protein tyrosine kinase; GPI, glycosylphosphatidylinositol; Vn, vitronectin; EC, endothelial cell; NFκB, nuclear factor kappa B; TGFβ, transforming growth factor beta; STAT, signal transducer and activator of transcription.

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In addition to mediating proteolysis and favoring invasion of tumor cells and tumor spreading, signaling via uPA–uPAR thereby mediating cell proliferation [9,10] or migration [11–13] would be one alternative. The uPAR/uPA/PAI-1 system is also involved in VEGF induced angiogenesis [14,15] contributing to tumor progression. Clinically, however, in several tumors the most striking correlation is found between levels of plasminogen activator inhibitor 1 and the malignancy of the respective tumor. High levels of PAI-1 shut off plasminogen activator dependent proteolysis making it unlikely that increased malignancy is simply due to increased local proteolysis. This again necessitates an alternative explanation.

## 2. Clinical data

A large body of clinical data exists on the correlation of components of the fibrinolytic system with tumor progression and adverse outcome. Specifically for the correlation with plasminogen activator inhibitor 1 and bad prognosis data are published in breast, ovarian cancer, gastric, colorectal, non small cell lung, renal cell and head and neck cancer as well as in brain tumors.

### 2.1. Mammary carcinoma

In breast cancer, PAI-1 antigen levels in tissues from 70 different patients were found to be significantly higher in patients who suffered a relapse [16]; similarly, in 196 patients with lymph node-negative primary invasive breast cancer PAI-1 ( $p=0.0015$ ), as well as uPA ( $p=0.0156$ ) had a significant impact on relapse-free survival [17]. In 576 patients with lymph node-negative breast carcinoma uPA–PAI-1 complex levels correlated with adverse histological grade and were found to be an independent predictor for overall survival ( $p=0.039$ ) [18]. A long-term follow-up of 276 patients demonstrated that in a node-negative subgroup PAI-1 levels in the primary tumor tissue was the only significant factor for disease free survival and the strongest factor for overall survival next to grading [19]. This data demonstrating a significant correlation between high PAI-1 levels in tumor specimen and adverse clinical outcome was confirmed further in larger studies: In 2780 patients with primary invasive breast cancer uPA and PAI-1 were found to be independent predictive factors of a poor relapse free and a poor over all survival in node-negative as well as in node-positive patients [20]; in 438 breast cancer patients followed for a median time of 10.3 years, high PAI-1 in tumor specimen was demonstrated as marker of poor prognosis. This study further suggested that the prognostic impact of PAI-1 is independent of its supposed involvement in tumor angiogenesis [21]. However, a contribution of effects of PAI-1 on the vasculature cannot be excluded to account for the adverse effects of high PAI-1 levels to “adverse clinical outcome”: In 136 well-characterized invasive breast carcinomas a significant correlation was observed between PAI-1 and vessel remodeling, patient age, nodal status and tumor grade, but no association with tumor vascularity was found, thus PAI-1 may be a key regulator of vascular remodeling in human breast cancer [22]. Furthermore, high PAI-1 levels in tumor extracts might also have an impact on response to therapy: PAI-1 in cytosolic extracts derived from primary breast tumors of 235 tamoxifen naive patients who had recurrent disease and who received tamoxifen therapy upon relapse (median follow-up was 57 months) negatively predicted response rates, which were better for patients with PAI-1-negative tumors than for those with PAI-1-positive tumors [23]

These data clearly indicate that high PAI-1 levels in tumor extracts of primary breast carcinomas correlate with adverse clinical outcome leading to the following concluding statement in a

review by Duffy [24] that “uPA and PAI-1 are among the first biological prognostic factors to have their clinical value validated using level of evidence 1 (LOE-1) studies. Determination of these analytes may help identify low-risk node-negative breast cancer patients for whom adjuvant chemotherapy is unnecessary”. He also states that: “paradoxically, high concentrations of plasminogen activator inhibitor (PAI-1), an endogenous inhibitor of uPA, also correlate with poor prognosis”. Therefore, determining PAI-1 levels in tumor extracts of primary breast cancers seems to be a good choice to predict possible clinical outcome for these patients.

### 2.2. Ovarian

Similar to breast cancer, high PAI-1 levels in tumor extracts seem also to be a prognostic marker in ovarian cancer: In 86 patients with advanced ovarian cancer FIGO stage IIIc, PAI-1 levels were found to be a strong, independent, prognostic parameter for overall survival [25] and in 103 ovarian cancer patients, elevated levels of PAI-1 ( $>18.8$  ng/ml) were associated with a shortened progression-free survival but not with malignant progression [26]. Similarly, in 131 patients with epithelial ovarian cancer, in which ~50% of the primary tumors and the metastases expressed PAI-1 among the patients with invasive stages III and IV, those whose primary tumors expressed PAI-1 had a shorter overall survival indicating that PAI-1 expression in the primary tumor epithelium is an independent poor prognostic factor for survival [27]; PAI-1 was also found as a predictor for overall survival in 70 patients with ovarian cancer but not as strong as stage and correlated with an unfavorable prognosis [28].

Although clinical evidence for a correlation between high PAI-1 levels and bad prognosis is not as strong in ovarian cancer as for patients with breast cancer, there is further additional data strengthening such a notion: PAI-1 concentrations correlated with the grade of differentiation in 104 ovarian cancers [29]; higher PAI-1 concentrations in cystadenomas, tumors with low malignant potential, as well as primary tumors of advanced ovarian cancer were consistently found in cancers with high pro-MMP-9 expression and increased with growing malignant potential of ovarian tumors [30]; patients with high grade tumor, recurrence and lower epithelial growth factor receptor (EGFR) and cathepsin-D had significantly higher PAI-1 levels as compared to those of the other patients ( $P<0.05$ ).

### 2.3. Gastric

In gastric cancer, in 180 patients disease-free survival was independently predicted by PAI-1 and cathepsin D and high expression of PAI-1 defined a subgroup of patients with a very bad prognosis [31]. In a consecutive prospective study of 203 gastric cancer patients (median follow-up 42 months) in addition to surgical curability, pT stage, pN stage, PAI-1 as well as c-erbB-2 were found to be independent prognostic factors for overall survival of curatively resected patients [32]. Furthermore, PAI-1 correlated with tumor size, lymph node involvement, differentiation and vascular invasion in 101 patients with gastric cancer [33]. In contrary, immunohistochemically assessed uPA and PAI-1 in specimens obtained from 105 gastric cancer patients revealed no statistically significant association of uPA levels with pT and pN, grading, depth of tumor invasion, UICC classification and the Lauren classification, while PAI-1 expression also exhibited no statistically significant correlation with pT, pN and M category or grading, but the UICC classification was significantly correlated with PAI-1 [34].

Taken together, data on PAI-1 and gastric cancer are not enough clear and abundant to allow a conclusion of a correlation between PAI-1 and clinical outcome.

## 2.4. Colorectal

In colorectal cancer, plasma PAI-1 levels before surgery weakly correlated with CRP levels in 594 patients, both parameters showing a Dukes independent distribution. When these patients were followed for a median period of 6.8 years high levels of PAI-1 were found to be associated with poor prognosis and low levels with good prognosis. Serum CRP was found to be a Dukes independent prognostic variable and to identify a subgroup of curatively resected patients at risk for short survival [35]. In contrast, however, low plasma levels of PAI-1 together with low levels of tetranectin and elevated levels of soluble uPAR and CEA were found to be associated with a 2.43 fold increased risk in 567 patients with primary colorectal cancer [36]. On the other hand, in 308 colorectal cancer patients followed for up to 16 years patients with PAI-1 -675 5G/5G genotype had better survival than patients with 4G/4G or 4G/5G genotypes when they had Dukes' stage A or B tumors [37]. These data indicate that also in colorectal cancer an association between high PAI-1 and bad prognosis exists as additionally indicated in a review by Berger [38]. Colorectal cancer is, however, one of the few, where data on a correlation between plasma levels of PAI-1 and prognosis are available. In addition these clinical outcome analyses are supported by data demonstrating that overexpression of uPA, uPAR and PAI-1 in the tumor tissue was significantly associated with liver metastasis in colorectal carcinomas [39] and that in colorectal tumors both MMP-1 and PAI-1 correlated with pathology, i.e. Dukes' stage, differentiation, lymphatic or vascular invasion and tumor depth [40].

## 2.5. Head and neck

For head and neck carcinoma also some reports exist demonstrating a correlation between high PAI-1 tumor levels and bad prognosis. In 79 oral cancer cases uPA and PAI-1 content appeared to be strong independent prognostic factors for relapse-free survival in squamous cell cancer of the oral cavity [41]. In metastasis-positive squamous cell carcinoma of the esophagus, a significant increase in PAI-1 scores was observed and according to this study PAI-1 might be used as a new parameter for prediction of prognosis in such tumors [42]. SPARC/osteonectin as found to be a powerful independent prognostic marker for short disease-free interval and in combination with other extracellular matrix proteins such as PAI-1 and uPA, the association with disease free interval and overall survival became even more significant ( $p < 0.001$ ) [43].

## 2.6. Nervous system

No larger studies on fibrinolytic parameters tumors of the nervous system and clinical outcome are available. However, malignant brain tumors show consistently stronger PAI-1 immunohistoactivity than benign or low-grade tumors. PAI-1 positivity was also found confined to glomeruloid-shaped proliferative vessels seen in high-grade gliomas and metastatic tumors, suggesting that PAI-1 may be involved in angiogenesis [44]. In 59 adult gliomas high PAI-1 levels were strongly associated with high histologic grade and histologic necrosis, however Grade 3 tumors with low PAI-1 (100% 3-year overall survival rate) presented the same clinical outcome as the low-grade tumors [44,45]. Furthermore, a functional co-operation of tPA, uPA, PAI-1 and vascular endothelial growth factor might exist during glioma progression [46]. On the other hand, overexpression of PAI-1 was shown to inhibit glioma cell motility and invasion through extracellular matrix (ECM) components, like laminin and collagen, but does not inhibit tumor cell invasion in a three-dimensional invasion assay, simulating normal brain tissue that has a different extracellular matrix and composition of the interstitium [47].

Taken together, the impact of the fibrinolytic system in brain tumors is not as clear as in other malignancies, but still PAI-1 can be found localized in both tumor and tumor-associated endothelial cells as reviewed in [48].

## 2.7. Urinary tract

For renal cell carcinoma (RCC) u-PA, u-PAR and PAI-1 were found to be strong and independent prognostic factors predicting early relapse in 152 patients with RCC; especially by means of PAI-1, a high and low risk group for disease free survival could be discriminated [49]. Similarly, in a group of 106 consecutive surgically resected specimens from patients with RCC the expression of uPA, uPAR and PAI-1 but not of PAI-2 correlated negatively with cause specific survival. Tumor associated macrophage counts correlated only with PAI-1 suggesting that PAI-1 is an important regulator of tumor progression and survival by modulation of associated macrophages [50]. On the other hand, in 100 patients with primary renal adenocarcinoma cytosolic uPA and PAI-1 levels were not predictive of metastasis [51].

With respect to prostate cancer, one of the most frequently occurring malignancies in elderly men, most data exist on plasminogen activators specifically on uPA [52–55] and malignancy of the tumor, but only scarcely data on PAI-1 [56–58].

## 2.8. Others

The giant cell tumor of the bone (GCT) is a benign tumor with a significant tendency to recur locally and rarely to produce pulmonary metastases; IL-6, u-PA, u-PAR and PAI 1 genes were found amplified, respectively, in 7%, 5%, 8% and 12% of the total 92 cases suggesting that higher expression of these genes might correlate with the presence of lung metastases [59].

With respect to non small cell lung carcinoma (NSCLC), the concentration of PAI-1 seemed to be associated with the histological cell types in 147 cases of NSCLC and identified along with cathepsin B (A7.5) and uPA as significant prognostic factor [60].

## 3. Animal experiments

Most data on the effect of high PAI-1 levels on tumor cell biology in animals were derived from experiments using mice either made genetically deficient in components of the fibrinolytic system or transgenic for one of the components and transplanting respective suitable tumors into these mice ("modified environment"). Another group of experiments analyses the behavior of tumors in which the expression levels of components of the fibrinolytic system had been modulated (selection, genetically, virus mediated gene transfer) in immune compromised animals. In addition to monitoring tumor progression, invasion and metastasis formation, also the response of the host towards the transplanted tumors is analyzed such as the angiogenic or the immune response.

### 3.1. PAI-1 over-expression in HT-1080 (and other cell types)/PAI-1 deficiency

In very early descriptive studies, it was found that the production of u-PA and PAI-1 by human melanoma cell lines correlated with their ability to form spontaneous lung metastasis in nude mice thus suggesting a role for u-PA and PAI-1 in a relatively early stage of melanoma metastasis [61]. In a similar approach using HT-1080 fibrosarcoma cells selected for their metastatic potential that co-selected with high PAI-1 levels it was found that a monoclonal antibody to PAI-1 suppressed pulmonary metastasis formation of

intravenously injected tumor cells with high metastatic potential by inhibition of tumor cell lodgement in vessels [62]. These results were confirmed using a low metastatic/low PAI-1 expressing clone and stably transfecting it with PAI-1. Transfection with PAI-1 converted the low metastatic subclone into a high metastatic one [63,64]. In contrast, performing similar PAI-1 transfection experiments but using a prostate carcinoma cell line PC-3, PAI-1 overexpression resulted in reduced tumor size and reduced density of tumor-associated microvasculature by 22–38%, inhibition of lung metastases and liver metastases [57]. In the latter experiments, a major difference was however, that the nude mouse model used did not utilize an iv. tail vein route indicating that when derived from a local tumor PAI-1 could inhibit tumor invasion and metastasis formation but does not exclude that when the tumor has already entered the circulation PAI-1 could increase tumor lodgement and in turn distant metastasis formation. Consistent with this view are data obtained with HT-1080 cells transduced by an adenoviral overexpression system with either PAI-1 or PAI-2 and analysed for metastasis formation of the subcutaneously implanted cells in nude mice. PAI-1 and PAI-2 significantly reduced the incidence of lung metastasis formation [65]. Such effect of PAI-1 on reducing metastasis formation (by 68% in this report) seems to depend on both inhibitory activity towards uPA as well as on interaction with vitronectin as revealed by using respectively mutated PAI-1 molecules and adenoviral transduction of HT-1080 tumor cells [66].

From these data one can conclude that high PAI-1 expression by the tumor is beneficial as long as proteolysis dependent mechanisms are responsible for tumor progression (inhibition of invasion), but that high PAI-1 levels are deleterious whenever proteolysis independent or even proteolysis inhibited responses are operative (e.g. tumor cell lodgment).

With respect to the impact of the fibrinolytic system of the host on tumor malignancy, in a model where transgenic mice overexpressing murine PAI-1 were used, no effect of the host's PAI-1 was found on the metastatic potential of B16 melanoma cells [67]. In an other study using overexpression of PAI-1 and PAI-2 by gene transfer into the liver, only PAI-2 reduced the incidence of lung and brain metastasis [65]. The effect of PAI-1 deficiency was analyzed in a transgenic mouse model of metastasizing breast cancer. Primary tumor growth and vascular density were unaffected by PAI-1 status and PAI-1 deficiency also did not significantly affect the lung metastatic burden. These data suggest that plasminogen activation is not rate limiting for tumor vascularization and metastasis or that a functional redundancy between PAI-1 and other inhibitors of the uPA/plasmin system exists [68]. On the other hand s.c. implantation of murine T241 fibrosarcoma cells into uPA<sup>-/-</sup> or PAI-1<sup>-/-</sup> mice reveal lower proliferative and higher apoptotic indices and a different neovascular morphology as well as decreased growth rates of tumors in the gene-deleted mice [69].

These data indicate that the status of the host with respect to components of the fibrinolytic system, specifically PAI-1 has no obvious impact on tumor progression or metastasis formation.

### 3.2. PAI-1 and angiogenesis

With respect to the role of urokinase-type PA (uPA), uPA receptor (uPAR) and PA inhibitor-1 (PAI-1) in angiogenesis *in vitro* studies have led to the notion that normal capillary morphogenesis is dependent on a protease-antiprotease equilibrium as reviewed by Pepper [70]. However, when one analyzes the effects of PAI-1 deficiency and PAI-1 overexpression in angiogenesis models *in vivo*, clearly two distinct effects can be separated: high levels of PAI-1 seem to inhibit angiogenesis, whereas “normal” levels of PAI-1 seem to be required for normal angiogenesis [71–73].

For example, in a study using malignant keratinocytes transplanted into PAI-1<sup>-/-</sup> mice, PAI-1 deficiency of the host mice prevented local invasion and tumor vascularization [74]. Similarly when T241 fibrosarcoma cells were s.c. implanted into either uPA<sup>-/-</sup> or PAI-1<sup>-/-</sup> mice, only PAI-1<sup>-/-</sup> mice showed resistance to corneal neovascularization but primary tumor growth was significantly diminished in all deficient mice relative to wt mice [69]. Consistently, lack of PAI-1 completely abolished angiogenesis in aortic ring assays using vessels from gene-inactivated mice, an effect that could be restored by exogenous PAI-1 and was dependent rather on the anti-proteolytic activity of PAI-1 than on its interaction with vitronectin [71], supporting earlier data by this group using adenoviral gene transfer of PAI-1 mutants [75]. However, an orally active small molecule inhibitor of PAI-1 (PAI-039; tiplaxtinin) binding near the vitronectin binding site also prevented angiogenesis in an *in vivo* tumor angiogenesis (neovascularization Matrigel implant) model [76]. Also in a model using HaCaT derived from a squamous cell carcinoma of the skin, tumor vascularization and invasion was blocked by disturbing the balance of matrix protease activity caused by a lack of PAI-1 in the stromal cells of the knockout mouse hosts [77]. Deficiency of host PAI-1 was also found to reduce angiogenesis and tumor invasion in a model using Rag-1<sup>-/-</sup> or a nude background in which the PAI-1 gene was deleted and transplantation of malignant human skin keratinocyte cell lines was employed [78]. With respect to the cellular origin of PAI-1 necessary for neo-vascularization, PAI-1<sup>-/-</sup> mice grafted with bone marrow derived from wild-type mice were able to support laser-induced choroidal neovascularization but not skin carcinoma vascularization, thus identifying the different cellular origin of PAI-1 as molecular determinant of a local permissive activity for angiogenesis (BM-derived cells for choroidal and local PAI-1-producing host cells for tumor angiogenesis) [79].

With respect to the anti-angiogenic activity of supra-normal concentrations of PAI-1, exogenously added PAI-1 at therapeutic concentrations was found to be a potent inhibitor of basic fibroblast growth factor (bFGF)-induced angiogenesis in the chicken chorioallantoic membrane [80]. Similarly, exogenously applied recombinant PAI-1 inhibited angiogenesis not only in three different *in-vitro* models but also *in-vivo* in a SCID mouse model using human LNCaP prostate cancer cells (as inducers of angiogenesis) [81]. Also in a model where PAI-1 was transfecting into the human pancreatic cancer cell line SW1990 resulting microvascular density was decreased as compared to non-transfected tumor cells [82]. All these data are consistent with the notion of a dual role of PAI-1 in angiogenesis.

## 4. In vitro data

From these animal data it is evident that the impact of PAI-1 on tumor malignancy is not a mere consequence of its activity to inhibit plasminogen activation and in turn proteolysis. The different effects of high PAI-1 levels on metastasis formation depend on the route of tumor inoculation. Moreover, the pro-angiogenic effects of “normal” and the anti-angiogenic effects of “high” levels of PAI-1 point to additional mechanism of PAI-1 over and above “fine tuning” of the equilibrium of proteases and anti-proteases. What is the *in vitro* evidence for such additional or alternative mechanisms?

### 4.1. PAI-1 and adhesion/de-adhesion

One of such mechanisms could involve PAI-1 mediated change in adhesion properties of anchorage dependent cells. Vitronectin is an important component of the extracellular matrix mediating integrin [83,84] and non-integrin dependent adhesion [85,86] and

PAI-1 also being a major component of the extracellular matrix of e.g. cultured human mesangial cells [87] interacts with vitronectin [88,89]. Immobilized vitronectin binds both purified PAI-1 as well as PAI-1 contained in culture medium of human sarcoma cells [90].

Binding of PAI-1 to vitronectin was found to control cell-matrix interactions by regulating the accessibility of attachment sites for  $\alpha_v\beta_3$  on vitronectin that overlap with the binding site for PAI-1 [91]. Another mechanism involves the PAI-1 induced formation of vitronectin multimers that bind to the anti-adhesive protein osteonectin thereby inhibiting (endothelial) cell adhesion [92]. PAI-1, however, does not only interfere with integrin binding to vitronectin, but also competes with uPAR for the same binding site (somatomedin B domain) in vitronectin. PAI-1 also dissociates bound vitronectin from uPAR and detaches U937 cells from vitronectin matrix [93]. PAI-1 was, however, also found to interfere with adhesion of cells to other matrix proteins such as fibronectin and type-1 collagen, a mechanism that might involve PAI-1 binding to uPA present in uPA-uPAR-integrin complexes on the cell surface, pointing towards a further anti-adhesive mechanism of PAI-1 by disruption of uPAR-VN and of integrin-VN interactions [94]. Similarly, exogenous PAI-1 prevented HT1080 cell adhesion (IC<sub>50</sub> 180 nM) and promoted cell detachment from vitronectin while antibodies to PAI-1 in contrast to an antibody to uPA did not affect adhesion of HT-1080 cells to vitronectin [95]. A further mechanism by which PAI-1 might lead to cell detachment was recently reported, whereby PAI-1 regardless of its conformation or activity induced detachment of LnCAP cells, a mechanism that might involve downregulation of follistatin [56].

PAI-1, however, was found not only to detach cells from vitronectin containing matrices, but also to increase cell adhesion. PAI-1 promoted adhesion and spreading of human myogenic cells in a dose dependent manner requiring the presence of urokinase at the cell surface; this effect was inhibited by antibodies to PAI-1 but also by antibodies against  $\alpha_v\beta_3$  integrins [96] indicating the participation of such integrins in PAI-1 induced increased adhesion. PAI-1 also modulated adhesion of human MDA-MB-435 breast carcinoma cells: exogenously added PAI-1 inhibited cell adhesion to vitronectin but not to fibronectin while stably transfected PAI-1 stimulated adhesion to both matrix proteins likely involving integrins, because blocking antibodies to  $\beta_1$  integrin inhibited PAI-1 induced adhesion [97].

#### 4.2. PAI-1 and proliferation

PAI-1, similar to uPA [9,10] might also affect cell proliferation: PAI-1-deficient ECs exhibited enhanced rates of cell growth and increase in VEGF receptor-1 (Flt-1) mRNA [98]. On the contrary, vascular smooth muscle cells with increased PAI-1 levels derived from transgenic mice (SM22-PAI+) exhibited increased proliferation rate together with increased expression of NF $\kappa$ B and activation of ERK [99]. Data available on PAI-1 and cell proliferation are, however, not conclusive and might involve PAI-1 effects on uPA activity and/or cell attachment.

#### 4.3. PAI-1 and migration/motility

Much more abundant but still not completely conclusive data are available on PAI-1 and cell motility. PAI-1 in its active conformation could be shown to block smooth muscle cell migration, probably mediated by competing for the overlapping site in vitronectin between  $\alpha_v\beta_3$  integrin and PAI-1; consistently, formation of a complex between PAI-1 and plasminogen activators converting active PAI-1 to PAI-1 complexes resulted in a loss of PAI-1 affinity for vitronectin and therefore restored cell migration [91]. Similarly, adenoviral overexpression of active PAI-1 reduced HT-1080

tumor cell migration, an effect that only partially was dependent on vitronectin binding [66].

In contrast, in myogenic cells that can utilize PAI-1 as an adhesion matrix molecule, interference with uPAR-uPA-PAI-1 complex formation as well as interference with binding of the complex to its internalization receptor the lipoprotein receptor related protein-1 LRP-1 markedly decreased myogenic cell motility [100]. Also for HT-1080 fibrosarcoma cells and the human melanoma cell line BLM, it could be shown that antibodies to PAI-1 dose-dependently inhibited not only invasive but also the migratory potential [95], an effect that cannot be explained by interfering with proteolytic activities. On the other hand wild-type PAI-1 increased cell motility in chemotactic assays for human MDA-MB-435 breast carcinoma cells, while a non-protease inhibitory P14 (T333R) PAI-1 mutant did not [99]. Interestingly, in a microarray analysis of 22 non-small cell lung cancer cell lines, 16 genes were positively and 7 negatively associated with invasiveness: the gene with the highest positive association with invasion was the protease inhibitor PAI-1 [101], further strengthening the notion that PAI-1 effects on invasion are not mediated by modulating mere proteolytic activities. In a cutaneous healing model (epidermal monolayer scraping), injury site closure was inhibited by anti-sense down-regulation of PAI-1 synthesis (by 80–85%), or addition of PAI-1 neutralizing antibodies; these data strongly indicate that PAI-1 was required for efficient long-term planar motility of keratinocytes. In fact, PAI-1<sup>-/-</sup> keratinocytes exhibited a significant wound healing defect that was evident even within the first 6 h following monolayer denudation injury. Addition of active PAI-1 protein to PAI-1<sup>-/-</sup> keratinocytes could rescue the migratory phenotype [102].

Although data on the effect of PAI-1 on cell migration are by far not uniform, there seems to be strong evidence that PAI-1 rather favors cell migration at least in non-vitronectin dependent systems. Migration of cells on a predominantly vitronectin matrix might rather be inhibited by PAI-1, a phenomenon that might be related to PAI-1 effects on adhesion of cells to vitronectin.

#### 4.4. PAI-1 and (uPA-uPAR) signaling

Only few studies address the question about a direct role of PAI-1 in signaling. Some hints to the role of PAI-1 in regulatory mechanisms involving intracellular signal transduction are also found in reports assessing the involvement of PAI-1 related molecules (mainly plasminogen activators and their receptors). However in most cases the presence/absence of PAI-1 in the system was not explicitly indicated. Hypotheses of signaling events triggered by PAI-1 or respective ligand complexes subsequent to binding to their cell surface receptors include uPAR, the LDL receptor family and laterally interacting molecules such as integrins, CD222-IGFII-R, EGFR, PDGFR and others. The uPAR-uPA-PAI-1 signaling is for sure rather complex and not a simple ligand – receptor induced response [4] and might involve interference with uPAR-uPA signaling, integrin signaling and endocytosis related signaling. Therefore pathways modulated by PAI-1 might include ERK1/2, AKT/PI3K, and the JAK/STAT pathway.

For example, in MCF-7 cells the kinetics of ERK phosphorylation in response to uPAR ligation was altered by PAI-1 in such a way that ERK phosphorylation was sustained, although PAI-1 did not directly activate ERK. This effect of uPA-PAI-1 complexes might involve a modification of the cooperation between uPAR and the very low density lipoprotein receptor (VLDLR) leading to tyrosine phosphorylation of focal adhesion kinase and Shc and sustained association of Sos with Shc; in contrast, uPA alone caused only transient association of Sos with Shc [103]. This effect of uPA-PAI-1 complexes might be explained by revealing a cryptic high-affinity binding site within the PAI-1 moiety for VLDLR by complex formation of PAI-1

with uPA; such “signaling” site is not contained in PAI-2 that is still efficiently endocytosed via this receptor but does not induce sustained mitogenic signaling events through VLDLR [104]. Similarly uPA – PAI-1 complexes induced signaling via the MEK/ERK pathway in MCF-7 and MDA-MB-435 breast cancer cells in an uPAR and VLDLR dependent manner [105]. PAI-1 was also shown to prevent in a vitronectin-dependent manner the cooperation of  $\alpha_v\beta_3$  integrins leading to a decrease in insulin-induced AKT phosphorylation in NIH3T3 fibroblasts [106]. Further, both active and inactive PAI-1 were shown to activate the JAK/STAT pathway thereby stimulating cell migration in chemotaxis, haptotaxis, chemokinesis and wound healing assays. This signaling effect might as well involve LRP as mediator of the migration-promoting activity of PAI-1 [107].

These data indicate that in fact PAI-1 might be involved in cellular signaling and such activity might probably not be related to inhibition of uPA but might be caused by uPA induced unveiling of a buried VLDLR binding site in PAI-1, whereby the involvement of LRP-1 cannot be excluded. This notion is supported by data showing that LRP-1-deficient cells can be rescued with respect to regulation of Rac1 activity by VLDL receptor expression [108]. In these studies LRP-1 in a uPAR dependent fashion was shown to function as a major regulator of Rac1 activation and in turn cell migration.

#### 4.5. PAI-1 and increased malignancy: a model

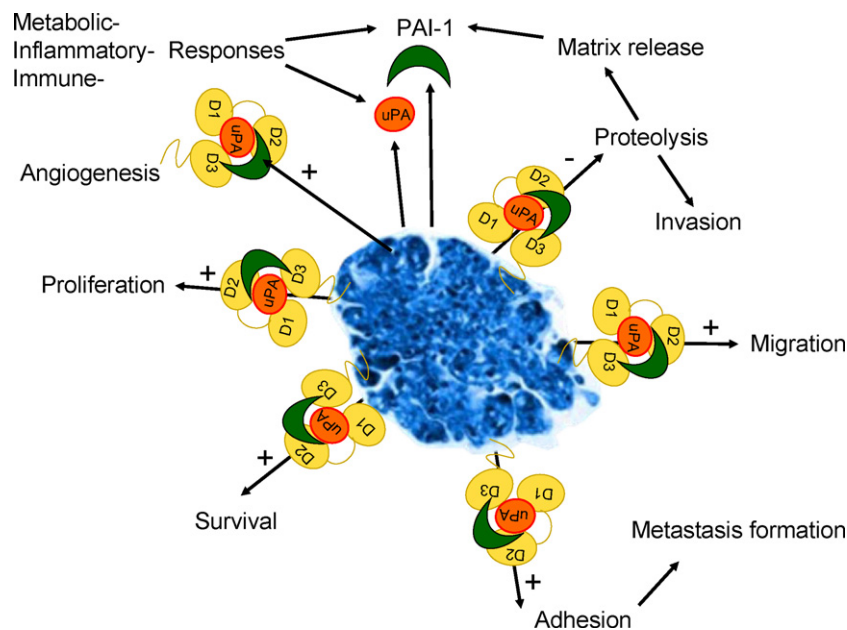
When summarizing the available reported data on PAI-1 in cancer, it becomes more and more clear that the clinical correlation between high PAI-1 levels and higher malignancy of the tumor reflected by “bad” clinical outcome is not in contradiction with data derived from animal experiments as well as *in vitro* data obtained in cells. In agreement with the complexity of the uPAR-uPA system that mediates focused proteolysis as well as signaling [109], the views on the role of this system in cancer are certainly changing [110,111]. It is now accepted that the system is also significantly involved in cell proliferation and angiogenesis and that “certain endogenous protease inhibitors such as PAI-1 and TIMP-1 appear

to promote cancer metastasis rather than inhibiting the process” [111].

However, what is the cellular basis for that correlation or is it just an epiphenomenon with or without a biological consequence and how can these data now be fitted into a model for a causal relation between high PAI-1 and increased malignancy of the tumor? It certainly cannot be a “single cause” model, but has to incorporate PAI-1 effects on several properties of the tumor as well as the host, e.g. increased angiogenesis.

##### 4.5.1. High PAI-1 levels as an epiphenomenon of more malignant cells

It was reported that the alkylating agent *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), induced p53 phosphorylation at Ser 15 that resulted in induction of PAI-1 in a p53-dependent fashion. This finding might link the tumor-suppressor protein p53 to high PAI-1 levels that could contribute to tumor metastasis [112]. In this case, high PAI-1 would be an “epiphenomenon with biologic consequence”. Another mechanism by which levels of PAI-1 would be increased in more aggressive tumors involves hypoxia: in patients with head and neck cancer PAI-1 levels measured in plasma correlated with the degree of tumor hypoxia leading to the hypothesis that changes induced by hypoxia at the transcriptional level could be responsible for a more aggressive phenotype. Up-regulated genes in these tumors included among others PAI-1 and related signal transducers such as LRP but also growth factors such as fibroblast growth factor-3, vascular endothelial growth factor, insulin-like growth factor-binding protein 3; PAI-1 mRNA gradually increased with hypoxia [113], but re-oxygenation could not revert high PAI-1 levels indicating that hypoxia “irreversibly” triggered pathways leading to PAI-1 upregulation [114]. Also in this case high PAI-1 levels would be an “epiphenomenon with biologic consequence”. Furthermore, TGF $\beta$  induction during the inflammatory response and in turn up-regulation of PAI-1 by active TGF $\beta$  [115] would also lead to the epiphenomenon “high PAI-1” as would also the metabolic syndrome [116]; in the latter case, breast cancer



**Fig. 1.** Target mechanisms potentially contributing to increased malignancy induced by PAI-1. The components of the basic signaling complex consisting of uPAR (yellow), uPA (red) and PAI-1 (green) are shown. Signal transducers such as LDLR members, integrins or G-protein coupled receptors are not shown. uPA mainly originates from the tumor and inflammatory cells; PAI-1 is released by the tumor, inflammatory cells, cells involved in PAI-1 secretion in the metabolic syndrome (e.g. adipocytes) as well as matrix cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

would be more aggressive in patients with metabolic syndrome. However, data obtained by transducing tumor cells with PAI-1 and experiments with PAI-1 deficient cells or animals indicate that PAI-1 secreted by the tumor can directly cause a more “malignant” phenotype.

#### 4.5.2. High PAI-1 “directly” causing a more “malignant” phenotype

From animal experiments as well as from *in vitro* data, the “pro-angiogenic” property of PAI-1 seems to be one of the prime candidates for the mechanism by which PAI-1 causes “bad” clinical outcome and therefore a more “malignant” phenotype in several cancers. This mechanism would represent the response of the host to PAI-1 secreted by the tumor. With respect to PAI-1 effects on the tumor cell, most data point towards a mechanism that involves ligation of PAI-1-uPA complexes to a member of the LDLR family, likely the VLDLR and LRP-1. Thereby a cryptic binding site in PAI-1 for the LDLR family member would be revealed by complex formation of PAI-1 with uPA and modulation of cellular signaling – initiated by uPAR – would occur. Signaling cascades thereby modulated include primarily the MAP kinase pathways as well as the JAK/STAT pathway and lead to a change in cell adhesion and migration.

Therefore (Fig. 1) PAI-1 secreted by tumor cells would stimulate in a paracrine fashion angiogenesis guaranteeing sufficient supply of the growing tumor with oxygen and nutrients and in an autocrine fashion would modulate cellular signaling leading to increased cell adhesion and migration reflected by increased metastasis formation. From this model, interference with PAI-1 signaling would be a promising approach for an alternative, additional tumor therapy.

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