

Regulation of programmed cell death by plasminogen activator inhibitor type I (PAI-I)

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Summary

Elevated levels of plasminogen activator inhibitor-I (PAI-I) are associated with poor prognosis in cancer. An explanation to the elevated levels of PAI-I could be a protective response to the increased proteolytic activity, caused by elevated levels of urokinase-type plasminogen activator (uPA) observed in tumours; however, several lines of evidence suggest that PAI-I may contribute directly to the pathology of the disease. PAI-I has been reported to have an effect on most of the basic cellular processes including cell adhesion, cell migration, cell invasion, and cell proliferation and increasing numbers of reports suggest that

PAI-I also can regulate programmed cell death (PCD) in cancer cells and normal cells. A number of reports suggest that PAI-I can inhibit PCD through its pro-adhesive/anti-proteolytic property whereas other reports suggest that PAI-I induces PCD through its anti-adhesive property. Furthermore, it has been suggested that PAI-I can either induce or inhibit PCD through activation of cell signalling pathways. This review will focus on the regulation of programmed cell death by PAI-I in both normal cells and cancer cells.

Keywords

Apoptosis, cancer, PAI-I, normal cells

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Introduction

Degradation of proteins in the extra cellular matrix (ECM) and the basement membrane is necessary for cancer cell invasion and metastasis. One of several enzyme systems involved in the disintegration of the ECM is the plasminogen activation (PA) system. Plasminogen activation is catalysed by at least two different serine proteases: urokinase-type (uPA) and tissue-type (tPA) plasminogen activators. uPA is primarily associated with tumour biology whereas tPA is associated with fibrinolysis in blood vessels. The major inhibitor of uPA and tPA is plasminogen activator inhibitor-1 (PAI-1). Based on the ability of PAI-1 to inhibit proteolysis, high PAI-1 levels in a tumour would be expected to inhibit cancer cell growth and dissemination in patients and therefore to relate to a more favourable prognosis. However, clinical studies have consistently demonstrated that PAI-1 is strongly related with poor outcome in cancer (1–4). The reason for this apparent discrepancy is still not fully understood, but still

more data demonstrate that PAI-1 is a multifunctional protein with various tumour-promoting characteristics. For example, PAI-1 has been shown to mediate/stimulate cell migration (5), to stimulate angiogenesis (6–8), and to modulate cell adhesion (9). Furthermore, it has been reported that PAI-1 can inhibit and promote programmed cell death (PCD) depending on the cell model (Table 1); however, the exact mechanism underlying PAI-1's apoptosis regulating function is still not known.

Molecular description of PAI-I

PAI-1 is a secreted 46 kDa single-chain glycoprotein containing 402 amino acid residues. PAI-1 belongs to the serine protease inhibitor (serpin) super-family but is unique among these because of its conformational and functional flexibility. Like other serpins, PAI-1 is meta-stable and exists in several different conformations including active, latent, cleaved, and complexed forms (10).

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Table 1: Regulation of PCD by PAI-1.

PAI-1	Model-system	Description	References
Inhibition of PCD	Cultured VSMC.	Inhibits PCD by binding to caspase-3.	(66)
	Cultured neurons.	PAI-1 from astrocytes protects neuron from PCD.	(60)
	PC12 cells.	Protects from PCD by induction of BclL and Bcl2.	(50)
	HUVEC, MCF-10A, HL-60, PC3 cells.	Inhibits chemotherapy, serum starvation and anti-Fas-induced PCD.	(48)
	Murine fibrosarcoma cells.	Inhibits PCD via activation of Akt.	(57)
	Aortic VSMC.	Inhibits plasmin-mediated PCD of VSMC.	(63)
	Primary lung fibroblast and IMR-90.	Inhibits plasmin-mediated PCD of fibroblasts.	(64)
Induction of PCD	Glial-neuronal co-cultures.	Inhibits IL-1 β -induced neurotoxicity.	(61)
	Vascular cells	Induces anoikis	(65)
	Primary fibroblasts	Induces PCD via inhibition of Akt	(71)
	In-vivo model, PC3 cells	Induces endothelial PCD in the newly established tumour vasculature.	(28)

PAI-1 forms a covalent 1:1 complex with either uPA or tPA. When the target protease is in its vicinity, PAI-1 forms a complex with it that distorts the active site of the protease and prevents its release from the serpin (11, 12). By abrogating the function of the plasminogen activators PAI-1 inhibits the conversion of plasminogen to plasmin, which ultimately leads to inhibition of fibrinolysis and degradation of the extracellular matrix.

If the complex of PAI-1:uPA is formed while the protease is attached to urokinase-type plasminogen activator-receptor (uPAR), it can be endocytosed after binding of α 2-macroglobulin receptor/LDL receptor-related protein (α 2MR/LRP) to uPAR (13). uPAR recycles to the cellular surface while the PAI-1/uPA complex is degraded (14). Alternatively, the very-low-density lipoprotein receptor (VLDLr) and the low-density lipoprotein receptor-related protein (LRP) can bind PAI-1 and mediate endocytosis of uPA:PAI-1 (15, 16). By mapping the VLDLr- and LRP-binding surfaces of the uPA-PAI-1 complex by site-directed mutagenesis, it was demonstrated that the affinity of the uPA-PAI-1 complex to VLDLr- and LRP was 10 to 100 times higher compared with the affinity of the free component suggesting an enhanced effect of bringing the binding areas of PAI-1 and uPA together on the same binding entity (17).

The internalization of uPA/PAI-1 has been associated with mitogenic signalling in a breast cancer cell line (MCF-7), and blocking of the binding of uPA/PAI-1 to VLDLr, either by treatment with a receptor-associated protein (RAP) or by using uPA in complex with a PAI-1 mutant incapable of binding VLDLr (uPA/PAI-1R76E) (18), results in decreased mitogenic signalling in these cells.

Interestingly, signalling through LRP has been suggested to be responsible for mediating survival in both Schwann cells (19) and in a breast cancer cell line (CL-16, derived from MDA-MB-435) (20).

In the absence of a protease, the active form of PAI-1 is unstable and undergoes transition to the latent form, which is ineffective as a protease inhibitor. Active PAI-1 circulates in the plasma and binds to the plasma-borne glycoprotein vitronectin. The transition of PAI-1 to latency is delayed by its binding to vitronectin. Both uPAR and free PAI-1, i.e. not bound to uPA, have high affinities for vitronectin, and the interactions of PAI-1 and uPAR with vitronectin are mutually exclusive.

Interestingly, PAI-1 can have opposing effects on the cell-adhesive properties of vitronectin. On one side, active PAI-1, but not latent or cleaved PAI-1, has the ability to bind vitronectin and block the binding of denaturated vitronectin to $\alpha_v\beta_3$ integrin in a dose-dependent manner thus leading to detachment of cells from the extracellular matrix (21). On the other side, PAI-1 cannot abolish pre-existing bindings between vitronectin and $\alpha_v\beta_3$ integrin; this leads to the suggestion that PAI-1 plays a role in modulation of the *de novo* cell adhesion (22). The ability of PAI-1 to inhibit vitronectin-dependent cell adhesion has furthermore been shown to inhibit migration of smooth muscle cells in different migration assays (23) and in alveolar epithelial cells (24). The interactions between PAI-1 and vitronectin have been attributed to binding to the somatomedin B (SMB) domain on vitronectin in a 1:1 relation (25). Recently, it has been shown that even if vitronectin lacks the SMB region it still retains PAI-1 binding capacity (26) due to the existence of a second binding site outside the SMB domain (27). This site has an approximately 100-fold higher $K(D)$ than the SMB site. Induction of PAI-1 expression *in vivo* leads to increased binding of vitronectin to PAI-1 followed by a relative displacement/uncoupling of integrin $\alpha_v\beta_3$ from vitronectin as shown in endothelial cells (EC) (28). Cells, which depend on integrin-mediated signalling for their survival, would therefore be expected to be sensitive to increased PAI-1 levels.

Programmed cell death

Programmed cell death (PCD; classic apoptosis, apoptosis-like cell death and necrosis-like cell death) is a cell suicide mechanism that enables multi-cellular organisms to maintain tissue homeostasis and to eliminate cells, which threaten the survival of the organism. De-regulation of apoptosis is observed in various diseases such as neurodegenerative diseases, where excessive cell death is pronounced, and cancers, where apoptosis is inhibited (29, 30). PCD can be triggered by a variety of stimuli including activation of cell surface death receptors (e.g. Fas, TRAIL-R1/R2, TNF-R1), anticancer agents, irradiation, and lack of survival factors (30–33). Even though the initial signalling pathways activated by various stimuli can be very different, the signalling cascades induced by most of them finally converge

into a common apoptotic pathway characterised by the activation of a family of cysteine proteases, the caspases. Although caspases are considered as main executioners in apoptosis, other proteases have also been suggested to play important roles in cell death (34). The signalling pathways that mediate PCD are tightly regulated by a delicate balance between pro-survival and death-inducing signals that determine if a cell will live or die (35). Generally, cell survival is controlled by a constant supply of survival signals provided by neighbouring cells (paracrine signalling) and by attachment to the ECM (36, 37). Accordingly, cells undergo PCD if the growth factors are withdrawn and/or if attachment of adherent cells to the matrix is prevented (anoikis). Both the growth factor receptors in the plasma membrane, which transduce the PCD-inhibitory signals into the cell, and the proteins that constitute the signalling pathways are targets of de-regulation during tumour-genesis. The phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway and the extracellular signal-regulated kinase (Erk)-signalling pathway are two major signalling pathways involved in various cellular functions including transmission of pro-survival signals (38, 39). Excessive signalling through the PI3K/Akt- and Erk-signalling pathways can be induced by growth/survival factors, and the signalling proteins in these pathways are frequently mutated or aberrantly expressed in human cancer. This leads to hyper-activation; thus suggesting that these survival pathways play an important role in tumour-genesis.

It is evident that the stromal microenvironment in which the cancer cells develop significantly influences several steps in tumour progression (40, 41). PAI-1-expressing cells in carcinomas of prostate, breast, and colon are primarily stromal myofibroblasts (42–44), and the fact that high PAI-1 levels in tumour tissue of these cancers are associated with poor prognosis suggests that PAI-1 expressed by the stroma promotes tumour growth and dissemination. In oral squamous cell carcinomas, PAI-1 is primarily expressed by the cancer cells (45), and high PAI-1 levels in oral cancer have also been associated with poor prognosis, thus indicating that PAI-1 can also stimulate tumour growth via an auto-crine loop (46).

The exact mechanism by which PAI-1 mediates protection against PCD is still not known, but PAI-1 may modulate cell survival/death through at least three hypothetical mechanisms: One mechanism is that PAI-1, through its anti-proteolytic activity, increases cell adhesion which may, in turn, increase pro-survival signalling. In support of this notion, PAI-1 has been shown to stimulate keratinocyte adhesion and to rescue keratinocytes from plasminogen-induced substrate detachment/anoikis (47). Another mechanism is that PAI-1 in complex with uPA/uPAR binds to LRP or VLDLr and induces pro-survival signalling into the cells. The third mechanism is PAI-1-mediated cell detachment/anoikis through binding of PAI-1 to vitronectin which prevents binding of vitronectin to $\alpha_v\beta_3$ integrin.

PAI-1 as a regulator of PCD in cancer cells

PAI-1 was first suggested to inhibit PCD in cancer in a study in which addition of recombinant PAI-1 to the human promyelocytic leukaemia cell line HL-60 and the human prostate carcinoma cell line PC-3 was shown to protect the cells against chemo-

therapy-induced apoptosis (48). In the same study, it was also shown that the protective effect of PAI-1 on spontaneous and camptothecin-induced cell death of HL60 cells could be reversed by a PAI-1 neutralizing antibody (MA-33B8), thus suggesting that the effect of PAI-1 addition was specific. This antibody induced a conformational change of PAI-1, rendering it unable to inhibit uPA, and thereby indicated that only PAI-1 in the active conformation can inhibit PCD.

The pro-survival function of PAI-1 has also been studied in the rat adrenal pheochromocytoma cell line PC12 (49–51). The PC-12 cell line is a polyclonal cell line which resembles sympathetic neurons when grown in the presence of nerve growth factor (NGF), and it has been used as an experimental model for sympathetic neurons (52, 53). Since PC-12 cells is a malignant cell line (54), the results from these studies may also reflect a pro-survival function of PAI-1 on cancer cells. It has been demonstrated that treatment of astrocytes with daunorubicin significantly lowered the PAI-1 expression level, and incubation of NGF-differentiated PC-12 cells with medium derived from daunorubicin-treated astrocytes did induce PCD. On the contrary, no change in morphology was observed in PC-12 cells incubated with untreated medium from astrocytes (49, 51). Addition of recombinant rat PAI-1 to the PAI-1-deficient medium protected the PC-12 cells against PCD, thus suggesting that PAI-1 has a pro-survival function in these cells. Furthermore, it has been shown that this apparent pro-survival function of PAI-1 involves a change in the balance between the mRNA expression levels of anti- and pro-apoptotic Bcl-2 family members (51).

The pro-survival function of PAI-1 has also been demonstrated in a cell model of murine wild-type and PAI-1 gene-deficient fibrosarcoma cells (55–57). By comparing the level of PCD induced by tumour necrosis factor (TNF)- α and a wide range of chemotherapeutic drugs, it was demonstrated that the PAI-1 expressing wild-type cells were significantly less sensitive to these drugs than the PAI-1 gene-deficient cells, thus suggesting a protective effect of PAI-1. Furthermore, when analysing the *in vivo* growth of tumours established from PAI-1 gene-deficient and wild-type cells it was shown that wild-type fibrosarcoma cells generated tumours much faster on nude mice than PAI-1 gene-deficient fibrosarcoma cells. Tumour growth was dependent only on the genotypes of the cell lines and not on the genotype of the recipient mouse (wild-type or PAI-1-deficient), and these *in vivo* data indicate that tumour growth depends on PAI-1 synthesised by the cancer cells rather than PAI-1 in the stromal compartment. Moreover, it was demonstrated that etoposide was equally toxic in wild-type and PAI-1 gene-deficient mice as judged by weight loss and white blood cell count (55).

Taken together, these data indicate that PAI-1 plays a role as a survival factor for cancer cells and that normal tissue, irrespective of their PAI-1 status, display equal sensitivity to chemotherapy. However, several lines of evidence demonstrate that PAI-1 also has a pro-survival function in normal cells.

PAI-1 as a regulator of PCD in normal cells

Several growth factors attenuate neuronal cell death induced by different types of injuries. The rescue of neurons by neurotrophins such as brain-derived neurotrophic factor (BDNF) in hy-

poxic-ischemic injury (58) and glial cell-line derived neurotrophic factor (GDNF) in N-methyl-D-aspartate (NMDA)-induced excitotoxic cell death are two illustrative examples. The role of PAI-1 as a neurotrophic factor has been suggested in a cellular model of NMDA-induced excitotoxic cell death; stimulation of mixed cultures of neurons and astrocytes where transforming growth factor- α and - β (TGF- α and TGF- β) induces a neuro-protective effect mediated through an up-regulation of PAI-1 in astrocytes by an Erk-dependent mechanism (59, 60). In another model of neuronal cell death, it has been shown that treatment of mixed cultures of neurons and glial cells with PAI-1 inhibits interleukin (IL)-1 β -induced neurotoxicity; suggesting that the plasminogen activator system contributes to IL-1 β -induced neuronal cell death (61). The consensus from these studies is that PAI-1 protein expressed by glial cells can reduce neuronal cell death in a paracrine fashion.

The plasminogen activator system and PAI-1 in particular, plays an important role in vascular pathology. Plasminogen activation has been suggested to contribute to apoptosis of vascular smooth muscle cells (VSMC) in atherosclerotic plaques (62) and furthermore, it has been suggested that PAI-1 is capable of impairing the plasminogen-mediated apoptosis of VSMC by inhibition of tPA or uPA (63). The role of PAI-1-mediated inhibition of plasminogen-induced apoptosis has also been demonstrated in a fibroblast cell model (64). TGF- β 1 was shown to up-regulate PAI-1 expression and TGF- β 1 also induced protection of fibroblasts from apoptosis induced by plasminogen in wild-type fibroblasts but not in PAI-1 gene-deficient fibroblasts. Stimulation of the fibroblasts with exogenously added PAI-1 protected the cells from plasminogen-induced apoptosis, thus indicating that TGF- β 1 inhibits apoptosis of fibroblasts via PAI-1 and inhibition of plasminogen activation. The PAI-1-mediated inhibition of plasminogen-induced apoptosis suggests that PAI-1 inhibits detachment of cells and, consequently, anoikis.

PAI-1 has also been reported to induce apoptosis of human umbilical-vein cells (HUVEC) and VSMC adherent to vitronectin but not fibronectin, and that this is correlated with PAI-1-mediated inhibition of cell adhesion (65). Contrary to this, addition of recombinant PAI-1 to HUVEC cells has been shown to inhibit PCD induced by serum-starvation (48). An explanation to this discrepancy could be that the regulation of PCD by PAI-1 depends on the surface the cells are grown on e.g. vitronectin, fibronectin or plastic.

Regulation of cell signalling pathways by PAI-1

Little is known about intracellular signalling pathways involved in PAI-1-mediated inhibition of PCD. However, in a model of VSMC apoptosis it was reported that elevated levels of PAI-1 in VSMC inhibit apoptosis via a direct interaction with and inhibition of the dominant death protease caspase-3 (66). This is a challenging result since it is the first reporting of an intracellular function of PAI-1.

It has been demonstrated in several studies that PAI-1 expression can either be up- or down regulated by the PI3K/Akt signalling pathway (67–70), but PAI-1 has also been reported to promote cancer cell survival by induction of the PI3K/Akt signalling pathway (57). In this study, it was demonstrated that

PAI-1 gene-deficient murine fibrosarcoma cells have a reduced Akt activity level when compared to the wild-type control cells. Re-introduction of the PAI-1 expression into the PAI-1-deficient cells induced an increase in Akt activation levels and conferred protection towards etoposide-induced apoptosis. Moreover, down-regulation of PAI-1 by siRNA in wild-type cells resulted in a reduction in Akt activation levels and an increase in sensitivity to etoposide-induced apoptosis. Contrary to this study, it has been demonstrated that wild-type aortic EC are more sensitive to EC growth factor withdrawal than PAI-1 gene-deficient aortic EC are (71). In this study it was further demonstrated that addition of exogenous PAI-1 to the PAI-1 gene-deficient EC resulted in an inhibition of the PI3K/Akt signalling pathway presumably by activation of the inhibitor of PI3K/Akt signalling, PTEN, and this was accompanied by an increase in apoptosis. In support of the role of PAI-1 as a negative regulator of the PI3K/Akt signalling pathway, it was demonstrated that down-regulation of PAI-1 in primary fibroblasts results in hyper-activation of the PI3K/Akt signalling pathway and bypass of replicative senescence downstream of p53 (72). This study further suggested that the negative regulation of the PI3K/Akt signalling pathway in primary fibroblasts may be accomplished by inhibition of uPA-mediated growth factor activation and release.

The conflicting data on whether PAI-1 induces or inhibits the PI3K/Akt signalling pathway could merely reflect differences in the regulation of this signalling pathway between the experimental cell models. Thus, PAI-1 may function as an inducer of the PI3K/Akt signalling pathway in cancer cells whereas PAI-1 may function as an inhibitor in normal cells (primary cells). The experimental set-up may also play an important role for the interpretation of the function of PAI-1, as demonstrated in a study by Palmieri et al. They showed that over-expression of PAI-1 in MDA-MB-435 breast cancer cells stimulated adhesion whereas exogenously added PAI-1 inhibited adhesion (9). This may lead to the consideration whether the spatial distribution of PAI-1 around a cell/cell layer is of major importance.

It has been suggested that the signalling of PAI-1 from the surface and into the cell is mediated through binding of uPA/PAI-1 complex to uPAR (18). In this study, uPA induces a transient activation of Erk-signalling in MCF-7 cells, whereas pre-formed uPA/PAI-1 complex is capable of inducing a sustained activation of Erk-signalling and induced proliferation in the MCF-7 cells. However, PAI-1 is not able to induce Erk-activation in MCF-7 cells by itself. The activation of the Erk-pathway by pre-formed uPA/PAI-1 complex is dependent on binding to uPAR and on the presence of the VLDLr; thus indicating that the uPAR-VLDLr complex transmits the mitogenic signal into the cell. In another study, binding of uPA to uPAR has been shown to induce the PI3K/Akt- and the Erk-signalling pathways resulting in increased Bcl-xL expression levels and protection from detachment-induced apoptosis of human retinal pigment epithelial cells (73). It would be interesting to study if stimulation with pre-formed uPA/PAI-1 complexes inhibit or enhance the pro-survival signal from uPA in this cell model.

Perspectives

The results from the large number of studies on the various functions of PAI-1 are conflicting, and the PCD-regulatory function of PAI-1 is no exception. The reason for this lack of consensus may be differences in the choice of experimental model and the experimental set-up in the models. However, the accumulating number of reports on PAI-1 and PCD suggest that PAI-1 primarily has a PCD-inhibitory function in cancer cells and exerts both PCD-inhibitory and -promoting functions in normal cells. The mechanisms involved in the transmission of the PCD-inhibitory and -promoting signals of PAI-1 have not yet been identified, but the use of PAI-1 variants can further elucidate this. One PAI-1 variant (PAI-1-R103A-M112A-Q125A), developed and described by Jensen et al. (74) has no measurable affinity to vitronectin but has intact uPA inhibiting capacity and LRP-binding capacity, and this protein can be useful in clarifying whether the death-promoting effect of PAI-1 is caused by PAI-1-mediated interference with the integrin-vitronectin binding. Likewise, the involvement of LRP-signalling in the pro-survival effects of PAI-1 could be further studied with a PAI-1 variant (PAI-1R76E), which is not capable of binding to LRP (75). The characterisation of this mechanism(s) is the major challenge for future studies on the biological actions of PAI-1.

The pro-survival function of PAI-1 in cancer cells could be a potential therapeutic target and may be useful in the development of new strategies in the treatment of cancer. Hence, inhibition of the pro-survival activity of PAI-1 by antibodies or small-molecular compounds could potentially sensitise the cancer cells to

subsequent chemotherapy. One limitation to this strategy would be if normal cells also respond to the potential sensitising effect of a PAI-1 inhibitor. In an *in vivo* PAI-1 knock-out mouse model, it was shown that the sensitivity of the animal to systemic treatment with etoposide, was unaffected by PAI-1 gene-deficiency, suggesting that mice lacking PAI-1 are not more sensitive to etoposide treatment than their wild type siblings (55). However, several studies based on models *in vitro* have demonstrated that PAI-1 is involved in regulation of PCD in normal cells; consequently, it is necessary to clarify whether sudden down-regulation of PAI-1 has other consequences for normal cells in general and as a response to chemotherapy, to determine whether inhibition of PAI-1 is a feasible strategy in treatment of cancer.

Abbreviations

BDGF, brain-derived neurotrophic factor; EC, endothelial cells; ECM, extracellular matrix; Erk, extracellular signal-regulated kinase; GDNF, glia cell-line derived neurotrophic factor; IL-1, interleukin-1; LRP, low-density lipoprotein receptor-related protein; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; PAI-1, plasminogen activator inhibitor type 1; PCD, programmed cell death; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; RAP, receptor-associated protein; TGF, transforming growth factor; TNF- α , tumour necrosis factor alpha; tPA, tissue-type plasminogen activator; TRAIL, TNF-related apoptosis-inducing ligand; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator-receptor; VLDLr, very-low-density lipoprotein receptor; VSMC, vascular smooth muscle cells.

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