Differential regulation of tumor necrosis factor α and transforming growth factor β production by the plasminogen activator inhibitor 1 in endothelial and cancer cells

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Abstract

The plasminogen activator inhibitor (PAI-1) is the key component of fibrynolytic system, its synthesis is rapidly activated by cytokines and mediators driving inflammatory response, both acute and persistent low-grade. While tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) are established potent direct stimulators of PAI-1 production in numerous tissues, little is known of any feed-back regulation that might be accountable for effects of PAI-1 on their synthesis. Present study provides evidence for the existence of negative feed-back regulation between PAI-1 and its potent activator TGF-β in endothelial cells. Dose-dependent inhibition of TGF-β production was observed in HUVEC cultures with physiological (10 μg/ml) and supraphysiological (100 μg/ml) ($p < 0.02$) PAI-1 concentrations exerting significant suppressive effect in comparison to control (respectively $p < 0.05$; $p < 0.02$) as well as cultures spiked with subphysiological PAI-1 dose of 1 μg/ml ($p < 0.0001$; $p < 0.02$). No such regulation was demonstrated for cancer cells of lung and prostate origin which might implicate different regulatory mechanism in neoplastic cells. Similarly, no functional interplay between PAI-1 and TNF-α levels in 24 hrs cultures of any evaluated cell lines was observed.

Key words: *PAI-1, TNF-α, TGF-β, HUVECs, lung cancer cells, prostate cancer cells.*

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Introduction

The plasminogen activator inhibitor (PAI-1) is considered a key regulator of plasminogen activator and consequently an important part of fibrynolytic system. Besides its obvious contribution to coagulation PAI-1 plays also considerable role in tissue proteolysis as well as other intracellular proteolysis-independent mechanisms. Consequently, it orchestrates cell adhesion and migration and therefore tumor initiation, proliferation, migration, invasion, metastasis formation and apoptosis. Likewise, due to its effect on endothelial cells PAI-1 partakes in new blood vessels formation, in both physiological and cancer-dependent angiogenesis [1, 2]. Importantly, PAI-1 is an early response protein known to be rapidly activated by cytokines driving inflammatory response, mostly tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β), but also by multiple trauma-associated stimuli like bacterial lipopolysaccharide (LPS), hypoxia and

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osmotic shock [3, 4]. It has been repeatedly demonstrated that cultured human cancer as well as endothelial cells considerably increase their PAI-1 production upon stimulation with TNF-α and TGF-β [5, 6]. Apparently, this phenomenon represents physiological regulatory mechanisms important for developing and sustaining inflammatory response in tissues as elevated circulating PAI-1 levels in blood has been observed in patients with severe acute infections, acute lung injury, as well as considerably milder but persistent systemic and local inflammation, obesity, atherosclerosis, pulmonary or renal fibrosis and invasive cancer [6-9].

The above mentioned numerous studies demonstrated direct stimulatory effect of TNF-α and TGF-β on the PAI-1 synthesis and its release to the local and systemic environment by different cell types. It has been shown that this effect is mediated primarily by p55 TNF-α receptor subunit and driven via activation of ERK, MAP and JNK kinases as well as NFκB transcriptional factors [5, 7, 10]. Mechanism of PAI-1 promoter activation by TGF-β has been attributed to the induction of Smad transcriptional factors but also MAP kinases and NFκB through the TGF-β activated kinase 1 (TAKI 1) [10, 11]. While the mechanisms responsible for stimulation of PAI-1 production by TNF-α and TGF-β are quite well established, information concerning feed-back regulation are scarce. The effects of PAI-1 on the TNF-α synthesis were studied in *in vitro* models of septic shock, while for TGF-β such evidence was provided by experiments with knockout animal models [12-15]. Therefore, to see whether PAI-1 affects in any way TNF-α and TGF-β production (positive or negative feed-back) we decided evaluate its effect in cultured non-LPS stimulated human endothelial and cancer cells of lung and prostate origin.

Material and methods

PAI-1 protein

The plasminogen activator inhibitor molecule characterized by the very long half-life of over 700 h (VLHL PAI-1) was constructed by the mutation of two amino acids (Gln197→Cys, Gly355→Cys), expressed in Sf9 cells, and purified as we described in details before [16,17]. Briefly, the cDNA encoding PAI-1 was excised from the VLHL PAI-1 plasmid as an *NdeI*/*XhoI* fragment. All mutations were introduced by PCR. The PCR product of the VLHL PAI-1s *NdeI*/*XhoI* fragment was ligated into the pFastbac plasmid, which contains a 6His purification tag. A bacmid containing VLHL PAI-1 DNA was used to transfect Sf9 cells derived from *Spodoptera Frugiperda* (Fall Armyworm, USA) using cell-lectin reagent (Invitrogen,USA), according to the manufacturer's instructions. The virus produced by the cells was then used to infect Sf9 cells. The flasks were incubated in a rotary incubator for 72 hrs at 27°C, harvested, and lysed by two freeze-thaw cycles. The lysate was centrifuged at 3000 × g for 20 minutes to pellet cellular debris. The supernatant was loaded onto a nickel resin-packed column (Invitrogen, Carlsbad, USA). The peak fractions were further purified on HPLC Superose 12 FPLC column (Millipore, Germany).

Cell lines culture

The human umbilical vein endothelial cell (HUVEC) line was purchased from Cambrex Inc., (East Rutherford, NJ, USA). Cells were grown to confluency in the EGM-2MV media (Cambrex Inc, USA) in an incubator (Sony, Japan); humidified atmosphere at 37°C, 5% CO_2. The prostate adenocarcinoma cell line LnCAP was propagated in RPMI 1640 medium, supplemented with 2 mM L-glutamine (Sigma, Poland). The human lung epithelium-derived cancer cell line NCI-H1299 (CRL-5803) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown with RPMI medium with 2 mM L-glutamine plus 10% fetal bovine serum and Antibiotic-Antimycotic (Gibco, Germany) in a humidified atmosphere at 37°C, 5% CO_2 (Sony, Japan).

TNF-α and TGF-β production assay

Cells were trypsinized in solution of 0.25% trypsin and seeded in quadruplicates into 24-well plates at the density of 10×10^4 cells/well. Cells were grown for 24 hours to the subconfluent state, subsequently fresh medium containing the different concentrations of VLHL PAI-1 of 1, 10, 100 μg/ml was added to wells. Treated cells were incubated for next 24 hours at 37°C, 5% CO_2. Afterwards supernatants were collected, centrifuged (1600 rpm, 10'), aliquoted and frozen in –80°C for further measurements.

Cytokine measurement was performed by quantitative enzyme immunoassay technique (ELISA) using commercial kits according to manufacturer recommendations (R&D) and was expressed as pg/ml. Minimal detectable concentration was 0.038 pg/ml (ultrasensitive) for TNF-α, while 4.61 pg/ml in case of TGF-β. Optical density was measured at 450 nm using spectrophotometer Elx800 (Biotek Instruments, Inc., USA).

Statistical analysis

Data were presented as a mean ($\bar{x}$) ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA test for mean values comparison. A value of $p < 0.05$ was considered significant.

Results

Increasing concentrations of mutated PAI-1 molecule (VLHL PAI-1) characterized by very long half-life time exerted no effect on the TNF-α levels in 24 hrs cultures of

any evaluated cell lines: lung cancer H1299, prostate cancer LnCAP, as well as human endothelial cells (HUVECs) (Tab. 1). Similarly, TGF-β production by cancer lines was not affected. However, dose-dependent inhibition of TGF-β production was observed in endothelial cells cultures. Both, physiological (10 μg/ml) and supraphysiological (100 μg/ml) PAI-1 concentrations exerted significant suppressive effect in comparison to control as well as cultures spiked with subphysiological PAI-1 dose of 1 μg/ml.

Table 1. The effect of VLHL PAI-1 on the TNF-α and TGF-β production by human lung cancer cells H1299, human prostate cancer cells LnCAP and human umbilical vein endothelial cells (HUVECs) assessed. Statistical difference was calculated by one-way ANOVA test for the results of cytokines production

Cell line	VLHL PAI-1 [μg/ml]	Cytokine production [pg/ml]	
		TNF-α $\bar{x}$ ± SD	TGF-β $\bar{x}$ ± SD
NCI-H1299	control	0.9 ±0.5	490.4 ±12.5
	1	0.7 ±0.5	494.5 ±55.8
	10	0.7 ±0.5	528.5 ±23.6
	100	0.3 ±0.2	514.8 ±52.8
LnCAP	control	0.5 ±0.3	382.0 ±65.9
	1	1.0 ±0.8	339.2 ±11.5
	10	0.7 ±0.7	442.0 ±22.2
	100	1.0 ±0.7	411.1 ±97.0
HUVEC	control	2.3 ±2.5	322.0 ±64.2
	1	1.7 ±2.1	337.0 ±64.6#
	10	2.1 ±2.5	128.0 ±2.7*## ‡
	100	2.0 ±2.6	20.4 ±0.6**

*vs. control * p < 0.05, ** p < 0.02,*
vs. cultures with VHLH PAI-1 100 mg/ml # p < 0.02, ## p < 0.001,
vs. cultures with VLHL PAI-1 10 mg/ml ‡ p < 0.05

Discussion

The plasminogen activator inhibitor promoter contains several regulatory elements which bind transcription factors (SBE, CAGA box, HRE, ERE, NFκB – binding sites, Sp-1, AP-1) and therefore allow inflammatory cytokines to regulate its synthesis. It has been also demonstrated that PAI-1 modulates several signaling pathways including ERK1/2, AKT/PI3K and JAK/STAT that participate in complicated network regulating TNF-α and TGF-β synthesis [6]. However, data concerning PAI-1 feed-back regulation of its two potent inducers TNF-α and TGF-β are very limited. Few studies directly targeting existence of such regulation between TNF-α and PAI-1 are mostly designed to evaluate PAI-1's potential effect on the rapid LPS-stimulated cytokine synthesis and production exemplifying acute stress/inflammatory reaction. Sitrin *et al.* examined the PAI-1 effect on TNF-α LPS-driven secretion by mononuclear phagocytes and demonstrated that while HMW uPA amplified cytokine production, PAI-1 suppressed it by ~ 47%. It was shown that uPA fragment containing the catalytic domain also effectively blocked TNF-α secretion, uPA receptor-binding domain had no effect, while TNF-α secretion was unaffected by plasminogen depletion or plasmin inhibition, therefore suggesting the non-fibrynolytic-dependend regulatory mechanism [13]. Beneficial role of PAI-1 in restraining the injurious effects of TNF-α expression in acute inflammatory diseases has been further confirmed in the animal model of acute pyelonephritis. It has been shown that bacterial infection caused significant increase of PAI-1 and PAI-1 directed neutrophil influx in kidneys of control mice while TNF-α, IL-1b and IL-6 levels were considerably lower in comparison to PAI-1 knockouts [18]. On the contrary to above, Kwak *et al.* reported that PAI-1 enhanced LPS-stimulated TNF-α and IL-β production by neutrophils acting via JNK-mediated pathway. Interestingly, uPAR involvement was not required and PAI-1 showed additive effects when combined with urokinase suggesting involvement of non-proteolytic pathways in this phenomenon [12]. Indeed, the same group has recently demonstrated that domains in the uPA molecule responsible for its proinflammatory effects (kringle domain) and receptors involved in uPA-induced cell activation are distinct from those responsible for its fibrinolytic and chemotactic properties (proteolytic domain). Thus, authors concluded that $\alpha_v\beta_3$ integrins rather than uPAR are involved in the uPA-driven up-regulation of the LPS-induced TNF-α production [19] which is in accordance with the previous studies. To provide more detailed explanation of this phenomenon, it should be reminded that PAI-1 acts in a complicated network of proteolytic cascade consisting of plasminogen, its active form plasmin, plasminogen activators [urokinase (uPA), tissue plasminogen activator (tPA)] and other activators inhibitors (PAI-2, nexin). PAI-1 down-regulates uPA fibrynolytic activity and triggers its half-life shortening (i) by binding with high affinity to the uPA complexed with its specific receptor (uPAR) on the cell membrane and (ii) by promoting endocytosis of uPA-uPAR-PAI-1 cluster through the low density lipoprotein receptor-related protein (LPR receptor) and its subsequent degradation [1]. Apart from its proteolytical activity PAI-1 interacts with vitronectin in a highly – specific manner, competing for binding with uPAR and integrins. It also disrupts existing uPA-uPAR-vitronectin complexes as well as induces internalization of uPA-uPAR- integrin clusters [20]. Therefore, uPAR-uPA-PAI-1 signaling is rather complex, not a simple ligand-receptor induced response and might involve interference with uPAR-uPA signaling, integrin signaling and endocytosis-related signaling.

Consequently, in view of the above mentioned contradictory experimental data and irrespective of the actual mechanism attributed to the uPA stimulatory effect on the LPS-induced TNF-α production, actual role of PAI-1 remains to be elucidated.

Whereas data concerning PAI-1 effect on TNF-α production in acute conditions seem to be inconclusive, we have demonstrated that in a non-acute setting of non-stimulated cell cultures, of both structural (endothelial cells) as well as malignant cells (lines originating from prostate and lung cancers), PAI-1 exerted no significant feed-back on the TNF-α concentrations irrespective to cells origin. These results are in accordance with previous data that showed no effect of PAI-1 on the expression of proinflammatory cytokines, TNF-α and IL-1β, by neutrophils or macrophages [12, 13]. Interestingly, phosphorylation of JNK, putative transcription factor involved in TNF-α regulation by PAI-1, was enhanced in resting neutrophils exposed to PAI-1 similarly as in LPS-stimulated cells [12]. That observation might suggest that while PAI-1 itself sustained JNK phosphorylation, it did not directly activate JNK. Thus, additional signals are necessary for the regulation of TNF-α promoter response by PAI-1.

The effect of PAI-1 on the production of TGF-β was evaluated in resting endothelial as well as cancer cells. Significant dose-dependent inhibition of TGF-β production was observed in HUVECs cultured with VLHL PAI-1, but not in prostate and lung cancer cell lines. There are several factors that might potentially be responsible for above outcome. It is well known that TGF-β is one of the most potent inducers of PAI-1 synthesis. However there is an increasing amount of evidence demonstrating that significant negative feed-back regulation exists between TGF-β and PAI-1, at least in some cell types. It has been shown that TGF-β_1 expression in the artery wall was increased 6- to 10-fold in vessels of mice lacking PAI-1 (*Serpine1*$^{-/-}$ mice) [21]. Similarly, in PAI-1 knockout embryonic mice fibroblasts TGF-β signaling and activity were significantly enhanced [14]. Our results from endothelial cell cultures are in line with above reports. Interestingly, inhibitory effect of PAI-1 in HUVECs was dose dependent which seems consistent with current views on mechanism regulating PAI-TGF-β axis. It is known that TGF-β activation might result from the direct cleavage of its latent form by serine proteases, such as plasmin, or by metalloproteases. Therefore, urokinase is considered the rate – limiting enzyme in the TGF-β_1 activation cascade. Importantly, the best described mechanism of TGF-β_1 activation is driven by integrins and modulated by proteases [22]. In particular, $\alpha_v\beta_3$ integrins pathway have been implicated in TGF-β regulation by PAI-1 [14]. It should be emphasized again that similarly to TNF-α, induction pathways for TGF-β production by resting cells are different from those initiated in these cells upon LPS stimulation [15]. Hence again, the rationale for experimental setting of our study.

Though it has been previously reported that TGF-β induces PAI-1 production in prostate and lung cancer cells, there are no data concerning feed-back regulation of TGF-β by PAI-1 in these cells [23, 24]. Similarly, little is known about potential mechanisms regulating their TGF-β secretion. However, it could not be excluded that final outcome, i.e. lack of suppressive effect of PAI-1 on TGF-β production in these cells might be due to the their unique urokinase secretory phenotype. Both cell lines, H1299 of lung cancer and LnCAP of prostate cancer are known for their high urokinase production. While urokinase plays key stimulatory role in TGF-β synthesis and activation, its excess might have also influenced PAI-1 effect by detaining it into uPA-uPAR-PAI-1 complexes.

In summary, our study provides evidence for the existence of negative feed-back regulation between PAI-1 and its potent activator TGF-β in endothelial cells, major tissue that synthesizes and secretes PAI-1 but also proinflammatory cytokines directly into the bloodstream, therefore allowing their systemic effects. No such regulation was demonstrated for cancer cells of lung and cancer origin which might implicate different regulatory mechanism in neoplastic cells. In accordance with other existing data, though quite limited, no functional interplay between PAI-1 and TNF-α non-stimulated cell cultures was observed.

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