Cell phenotype determines PAI-1 antiproliferative effect — suppressed proliferation of the lung cancer but not prostate cancer cells

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Abstract

Introduction: Plasminogen inhibitor activator type 1 (PAI-1) is an important regulator of tumor growth and metastasis formation acting directly via specific uronkase complexing or indirectly due to its affinity to vitronectin. We have shown previously that PAI-1 modifies angiogenic activity of endothelial cells in a dose-dependent manner but also in close relationship to the cell phenotype. Present study aimed on evaluating the PAI-1 effect on the proliferative activity of lung cancer cells (A549), prostate cancer cells (DU145) as well as endothelial cells (HUVEC).

Results: Mutated PAI-1 (1, 10, 100 μg/ml) characterized by the prolonged antifibrinolytic activity (T_{1/2} ~ 7000 h) inhibited proliferation of lung cancer A549 cells in a dose-dependent (p < 0.001) and time-dependent (p < 0.001) manner. No significant effect on the DU145 prostate cancer cells has been observed except of the 72 h cultures with highest PAI-1 concentration (100 μg/ml) (p < 0.001). Proliferative activity of endothelial cells (HUVEC) was affected by 100 μg/ml PAI-1 only, and independent of the culture period (24, 48 and 72 h, p < 0.001).

Conclusion: Plasminogen inhibitor activator type 1 modulates cell proliferation via antifibrinolitic mechanism time- and dose-dependently, however final outcome is strongly affected by the cell phenotype.

Key words: plasminogen activator inhibitor type 1, lung cancer, prostate cancer, endothelial cells

Introduction

Plasminogen activator inhibitor type 1 (PAI-1) is the most potent physiological inhibitor of plasminogen-activating kinases: urokinase (uPA, urokinase plasminogen activator) and tissue plasminogen activator (tPA). It is essential for the maintenance of physiological balance between the coagulation and fibrinolysis systems in vivo [1]. Abnormal PAI-1 expression is found in many diseases and plays a key role in the development of cancer [2, 3]. Many years of studies investigating the role of PAI-1 in these processes have led to a better understanding of the complex nature of its involvement in tumour growth and its interactions with the surrounding tissues [4]. As already mentioned, PAI-1 is the most potent and highly specific inhibitor of urokinase, which plays a key role in migration and invasiveness of tumour cells, determining the increased plasminogen activity on their surfaces and initiating the proteolysis cascade that leads to the degradation of bonds between...
extracellular matrix proteins and the loss of intercellular contacts [5]. By exerting its action in combination with its specific surface receptor (uPAR, urokinase plasminogen activator receptor), urokinase acquires a unique chemokine-like property, comprehensively regulating not only cell migration but also the related processes of cell shape change, adhesion, chemotaxis, and invasion of the extracellular matrix. PAI-1 directly suppresses the proteolytic activity of uPA and its chemokine-like and adhesin-like effects and considerably shortens the half-life of this protein. Through competitive interaction with vitronectin it also inhibits interaction with the specific receptor, which leads to the suppression of urokinase-dependent adhesion and migration [1]. An important effect of PAI-1 on these processes is observed under physiological homoeostasis, when it effectively blocks subsequent stages allowing cell migration within the tissues and triggers mechanisms that allow re-initiation of these processes, which guarantees the maintenance of a dynamic balance between them. The interaction between PAI-1 and uPA under these conditions is subject to constant changes, and its net result depends both on the quantitative predominance of one of these proteins and on the effects of other biological factors.

In our previous reports, we demonstrated that the biological activity of PAI-1 was strictly related to its concentration in the environment studied (tissue, cell cultures) but also to the half-life, which indirectly reflects the antifibrinolytic activity of PAI-1 or its ability to react with uPA in time. The ability of PAI-1 to affect the process of vascular bud formation was directly proportional to its half-life. A mutated protein with a considerably extended antifibrinolytic activity exerted a potent dose-dependent inhibitory effect, while the wild protein, the half-life of which is short, also stimulated angiogenesis in a dose-dependent manner in the experimental system [6–8].

The aim of our study was to evaluate the significance of these elements, the half-life, and the concentration, on the modulating effect of a mutated PAI-1 form characterised by a considerably extended antifibrinolytic activity (T1/2 = 7000 hours) relative to the proliferative activity of various tumour cell lines (lung cancer and prostate cancer cell lines in this case) and vascular endothelial cells [6].

**Material and methods**

**Cell cultures**

We cultured human non-small cell lung carcinoma cells A549 (CCL-185), prostate cancer cells DU145 (ATCC, Manassas, VA, USA), and human umbilical vein endothelial cells (HUVEC) (Cambrex Inc., East Rutherford, NJ, USA) until a confluent growth was achieved in the culture media. We used Ham F12 (Sigma) or RPMI 1640 (Sigma) with the addition of 2 mM of L-glutamine (Sigma), 10% foetal bovine serum (FBS) (Sigma), and antibiotics (Antibiotic-Antimycotic, Gibco) for the cancer cells and EGM-2MV (Cambrex Inc., USA) for the HUVEC cells, in a culture chamber at 37°C and humid atmosphere with 5% CO2 (Sony, Japan).

The cells were washed with phosphate-buffered saline (PBS) without Ca2+ and Mg2+ and trypsinised in a 0.05% trypsin solution (Gibco), cell density was determined by haemocytometry, and the cells were cultured into 96-well plates at 4000 cells/well (A549, DU145) or 5000 cells/well (HUVEC). At 48 hours of culture, after confluent growth had been achieved, the medium was changed and PAI-1 was added at concentrations of 1, 10, and 100 μg/ml in triplicate. The cultures were maintained for 24, 48, and 72 hours under standard conditions. Each experiment was repeated three times.

**Proliferative activity of the cells**

The proliferative activity of the tumour cells was assessed at 24, 48, and 72 hours after addition of PAI-1, using a commercial EZ4Y cytotoxicity assay (Bio-medica). The assay was performed to the manufacturer’s instructions. Briefly, following the addition of the substrate at the quantity corresponding to 10% of the culture media volumes and incubation at 37°C and 5% CO2 for 2 hours, the plates were shaken for 5 minutes at 300 rpm, and absorbance at the wavelength of 450 nm was measured (Infinite M200, Tecan).

The proliferative activity of endothelial cells was assessed at 24, 48, and 72 hours following the addition of PAI-1, using an In Vitro Toxicology Assay Kit Neutral Red Based (Sigma-Aldrich, Poland). The assay was performed to the manufacturer’s instructions. Following the addition of neutral red at the quantity corresponding to 10% of the culture media volumes and incubation at 37°C and 5% CO2 for 2 hours, the medium was removed and the cells were washed with a fixing reagent and treated with 100 ml of a dissolving substance. Absorbance was measured at the wavelength of 540 nm (Infinite M200, Tecan).

The results were summarised as a percentage of the control (the control consisted of cells growing in a standard culture medium appropriate for the cell line without the addition of PAI-1).

**Results**

The mutated PAI-1 form, which is characterised by considerably extended antifibrinolytic ac-
Table 1. The effects of mutated PAI-1 with a very long half-life (PAI-1 VLHL) on the proliferative activity of the A549 lung cancer cell line and the DU145 prostate cancer cell line

<table>
<thead>
<tr>
<th>PAI-1 VLHL [μg/ml]</th>
<th>Proliferation versus control (%)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td>A549</td>
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<tr>
<td>1</td>
<td>105,67 ± 5,53</td>
<td>101,81 ± 3,02&lt;sup&gt;2&lt;/sup&gt;</td>
<td>92,26 ± 3,02&lt;sup&gt;1,4&lt;/sup&gt;</td>
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<tr>
<td>10</td>
<td>95,45 ± 1,38&lt;sup&gt;1&lt;/sup&gt;</td>
<td>90,82 ± 3,55&lt;sup&gt;1&lt;/sup&gt;</td>
<td>69,68 ± 4,40&lt;sup&gt;1,3&lt;/sup&gt;</td>
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<tr>
<td>100</td>
<td>86,42 ± 2,43&lt;sup&gt;2,2&lt;/sup&gt;</td>
<td>70,62 ± 1,19&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>53,68 ± 3,09&lt;sup&gt;2,3,4&lt;/sup&gt;</td>
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<td>DU145</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>103,54 ± 5,28</td>
<td>98,00 ± 1,33</td>
<td>102,64 ± 0,72</td>
<td></td>
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<tr>
<td>10</td>
<td>99,73 ± 1,06</td>
<td>96,66 ± 2,31</td>
<td>102,24 ± 5,57</td>
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<tr>
<td>100</td>
<td>97,00 ± 5,19</td>
<td>93,43 ± 5,52</td>
<td>91,18 ± 5,10&lt;sup&gt;3,5&lt;/sup&gt;</td>
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</table>

<sup>1</sup>p < 0.001 versus cells cultured in the presence of PAI-1 at 1 μg/ml; <sup>2</sup>p < 0.001 versus cells cultured in the presence of PAI-1 at 10 μg/ml; <sup>3</sup>p < 0.001 versus a 24-hour culture; <sup>4</sup>p < 0.001 versus a 48-hour culture; <sup>5</sup>p < 0.01 versus cells cultured in the presence of PAI-1 at 10 μg/ml; SD — standard deviation

Table 2. The effect of mutated PAI-1 with long half-life time (PAI-1 VLHL) on the proliferation activity of human endothelial cells

<table>
<thead>
<tr>
<th>PAI-1 VLHL [μg/ml]</th>
<th>Proliferation versus control (%)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td>HUVEC</td>
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<tr>
<td>n = 9</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>96,95 ± 3,93</td>
<td>99,16 ± 3,38</td>
<td>93,25 ± 7,65</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>89,06 ± 1,67</td>
<td>92,39 ± 7,15</td>
<td>91,25 ± 7,95</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>79,52 ± 8,00&lt;sup&gt;1,4&lt;/sup&gt;</td>
<td>79,69 ± 3,45&lt;sup&gt;1,3&lt;/sup&gt;</td>
<td>74,64 ± 12,05&lt;sup&gt;4&lt;/sup&gt;</td>
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</table>

<sup>1</sup>p < 0.001 versus cells cultured in the presence of PAI-1 at 1 μg/ml; <sup>2</sup>p < 0.01 versus cells cultured in the presence of PAI-1 at 1 μg/ml; <sup>3</sup>p < 0.001 versus cells cultured in the presence of PAI-1 at 10 μg/ml; <sup>4</sup>p < 0.01 versus cells cultured in the presence of PAI-1 at 10 μg/ml; SD — standard deviation

tivity, significantly suppressed the proliferative activity of the A549 lung cancer cells with increasing concentration in the culture medium (Table 1). The proliferative activity of the A549 cells cultured in the presence of 100 μg/ml of PAI-1 was significantly lower compared to the culture with the addition of 1 μg/ml of PAI-1 (p < 0.001) as early as at 24 hours. The inhibitory effect of PAI-1 increased with time and was significantly higher at 48 and 72 hours of culture.

In the cultures of the DU145 prostate cancer cells we observed no dose- or time-dependent inhibition. Only the cells exposed to the highest concentration of PAI-1 (100 μg/ml) for 72 hours showed any reduction in proliferative activity compared to the other cultures (1, 10 μg/ml) (p < 0.001).

The proliferative activity of human umbilical vein endothelial cells was significantly suppressed by PAI-1 at 100 μg/ml as early as at 24 hours. This effect was also observed in the next days of the culture at 48 and 72 hours (Table 2). The inhibitory effect of PAI-1 did not show dynamics in time.

Discussion

The tissue microenvironment in which tumour cells proliferate is characterised by considerable dynamics resulting from the imbalance of systemic and local functional homeostasis in the patient’s body and from the individual characteristics of these cells: the phenotype of the tumour. This is particularly the case with PAI-1, which is an element of an extremely complicated system of interrelations, very briefly referred to as actions of PAI-1 via uPA or via vitronectin [1]. In reality, interactions of proteins within this biological system are much more complex, and current knowledge provides us with a very limited understanding of the complexities of interactions at the protein and gene levels. This is reflected by significant inconsistencies in the published results of studies investigating the effects of PAI-1 on the angiogenic activity of tumour cells. While the potent stimulating effect of urokinase and its receptor is generally considered well supported, the role of PAI-1 in these processes is still debatable and the results of
experimental studies are inconsistent [9–13]. It seems that the reason for these inconsistencies is the high complexity of interrelations between PAI-1 and the remaining components of the plasminogen-plasmin system and the extremely variable reactions of other cytokines and mediators released by the cells according to their phenotypic characteristics [14]. One must also remember the numerous technical limitations of the in vitro and in vivo experimental models and the problems arising from the short half-life of the wild-type PAI-1 (T_{1/2} = 60 minutes) and the unsatisfactory stability of the modified forms of PAI-1 developed so far (maximum T_{1/2} = 145 hours) [15]. The very long half-life of the mutated PAI-1 form we developed (T_{1/2} ~ 7000 hours) eliminated some of the technical problems mentioned above and left no doubt about the activity of the PAI-1 protein in the culture or the mechanism by which it affects the cells.

We analysed the effect of PAI-1 on the proliferative activity of tumour cells (the A549 lung cancer cell line and the DU145 prostate cancer cell line) and observed a significantly different response to the presence of this protein in the culture microenvironment. The available literature lacks studies evaluating the effect of PAI-1 on the proliferative activity of the A549 cell line or other lung cancer cell lines. Indirect data come from studies which evaluated the effect of transforming growth factor β (TGF-β) on the proliferation of lung cancer cells and showed that the antiproliferative effect of this cytokine results from the initiation of PAI-1 release and disappears when its synthesis is inhibited [14]. The significant correlation that we observed between the concentration of PAI-1 and the duration of its effects on the degree of A549 cell proliferation suppression seems very interesting. This correlation convincingly confirms that interaction between PAI-1 and uPA (and hence the antiproliferative mechanism) is the key mechanism that regulates the proliferative activity of the A549 cells. The A549 cells are typical “manufacturers” of uPA, and the release of uPA is markedly stimulated by hypoxia [16]. This fact may be an interesting starting point for further studies as it should be assumed that the antiproliferative action of PAI-1 is significantly modified in the presence of excess uPA in the microenvironment (uPA and PAI-1 form a complex at a 1:1 ratio) [1].

In contrast to the lung cancer cell line, the DU145 prostate cancer cell line was characterised by a significant resistance to the long-acting mutated PAI-1 form. With the exception of the highest dose of PAI-1 at 72 hours of culture, no significant effect on the proliferative activity of these cells was observed. Although Soff et al. [10] did report inhibitory action of PAI-1 on the growth of the primary tumour and metastasis formation in mice after implantation of prostate cancer cells, subsequent studies failed to confirm that this effect was associated with a potent proapoptotic effect of PAI-1 on vascular endothelial cells and with an antiangiogenic effect [17, 18]. Also, the antiproliferative effect of PAI-1 on vascular endothelial cells observed by us is consistent with the cited reports on angiogenic effects of this protein mainly resulting from its antifibrinolytic action. Our findings are also consistent with other studies demonstrating an inhibitory effect of PAI-1 on the healing of vascular endothelium following surgery or abnormalities of in vitro endothelial cell proliferation [19, 20]. This analysis clearly confirms how very difficult studies investigating the biological role of PAI-1 are, emphasising the multiplicity of elements significantly affecting the biological effects of this protein. PAI-1 has been shown to modulate the proliferative activity of cells by inhibiting urokinase in a strictly dose- and time-dependent manner but also in a manner dependent on cell phenotype. This latter relationship indicates the necessity to take extreme care in interpreting studies evaluating cells of various origins.

References