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Malignant transformation of wild-type but not plasminogen activator inhibitor-1 gene-deficient fibroblasts decreases cellular sensitivity to chemotherapy-mediated apoptosis

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Abstract

Plasminogen activator inhibitor-1 (PAI-1) inhibits the activation of the plasminogen activator system, the latter being involved in cancer growth and dissemination. Interestingly, PAI-1 is elevated in many solid tumours and this elevation has consistently been shown to be associated with shorter length of patient survival. This study aims to determine whether PAI-1 contributes to cancer cell growth by inhibiting apoptosis of tumour cells. It is shown that spontaneous transformation decreases cellular sensitivity to chemotherapy-mediated apoptosis of wild-type, but not PAI-1 gene-deficient, fibrosarcomas. PAI-1 gene-deficient and wild-type mice displayed similar sensitivity to treatment with etoposide, suggesting a differential effect of PAI-1 expression between cancer cells and normal cells. Thus, since PAI-1 appears to be an important factor in regulating apoptosis in cancer cells but not in normal cells, inhibitors of PAI-1 might be useful as sensitising pre-treatment for subsequent apoptosis-inducing anti-cancer therapy.

Keywords: PAI-1; Cancer; Apoptosis; Plasminogen activator

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) belongs to the serpin (serine proteinase inhibitor) superfamily, which includes inhibitors of a variety of serine proteinases, e.g., PAI-2 and PAI-3. The PAI-1 gene encodes a ~50 kDa glycosylated protein, which is secreted from the cell. PAI-1 is the primary inhibitor of the plasminogen activation system, a proteolytic cascade involved in various physiological and pathological processes including wound healing, inflammation, vascular thrombolysis, tumour invasion and angiogenesis [1,2]. PAI-1 inhibits the two types of plasminogen activators, the tissue-type plasminogen activator (t-PA) and the urokinase-type plasminogen activator (u-PA). Both activators are capable of catalysing the conversion of the inactive zymogen plasminogen to the active proteinase plasmin, which can degrade most extracellular proteins; a mechanism involved in cancer dissemination. As an inhibitor of the plasminogen activation system one would expect high levels of PAI-1 to inhibit tumour progression. However, high levels of PAI-1 are correlated with poor prognosis in a number of tumours, including carcinomas of the breast, ovaries and stomach [3–6]. One explanation for this apparent discrepancy is that PAI-1 has a pro-angiogenic effect [7]. However, it has been suggested recently that the prognostic impact of PAI-1 is not based on its involvement in angiogenesis

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alone [8]. Another explanation is that high levels of PAI-1 contribute to tumour growth by inhibiting apoptosis of tumour cells. In support of this theory, it has been shown that addition of PAI-1 to tumour cells in culture inhibits apoptosis [9].

Apoptosis can be triggered by a variety of stimuli, including activation of cell surface death receptors (Fas, TRAIL-R1/R2, TNF-R1, *etc.*), anticancer agents, irradiation, lack of survival factors and ischaemia [10,11]. Even though the initial signalling pathways induced by various stimuli can be very different, the signalling cascades induced by most reagents finally converge into a common apoptotic pathway. The various functions of PAI-1 have been studied in depth [12], however, little is known about how PAI-1 regulates apoptosis. In order to study further the role of PAI-1 in apoptosis, we established PAI-1 gene-deficient and wild-type fibrosarcoma cell lines and tested the cells for sensitivity to induced apoptosis. Here, we show that PAI-1 might regulate apoptosis of transformed cells but not normal cells. Spontaneous transformation of fibroblasts decreased cellular sensitivity to chemotherapy-mediated apoptosis of wild-type, but not PAI-1 gene-deficient, fibrosarcomas. Furthermore, PAI-1 gene-deficient and wild-type mice display equal sensitivity to systemic etoposide treatment, thus suggesting a differential sensitivity between cancer cells and normal cells to apoptosis inhibition by PAI-1.

2. Materials and methods

2.1. Cell culture

Lungs of 10–13-week-old male Meta nu/nu mice of either wild-type or PAI-1 −/− background (see below) were excised and placed in a Petri dish with 10 ml media (M199 with 30% foetal calf serum (FCS), penicillin and streptomycin and 1% $NaHCO_3$). The lungs were cut mechanically into small pieces (≈0.5–1 mm^2). Then, 3–6 pieces were placed in each well of a 6 well plate (Nunc, Tissue culture Quality) in a drop of media (from the cutting) and placed in a CO_2 incubator at 37 °C for 20 min to allow the cells to adhere to the bottom of the well. After 20 min, 1 ml of media was added to cover the tissue completely. After another 30 min, another 1 ml of media was added. The media were renewed every 3 d. The wells were inspected at regular intervals and after three weeks they were changed to media without penicillin and streptomycin. After 4–5 weeks, wells with outgrowth of fibroblasts were harvested and the cells pooled and grown in CM (M199 + 10% FCS + 0.15% $NaHCO_3$). The cells were tested and found free from Mycoplasma contamination. Genotyping to confirm the origin of the cells was performed on a regular basis (see below) and the PAI-1 +/+ and PAI-1 −/− fibrosarcoma cells were named Pwt-I and Pko-I, respectively. Following a similar procedure, a second set of cell lines (Pwt-II and Pko-II) was established. All sets of +/+ and −/− cells were derived from litter-mates.

2.2. Compounds

Etoposide was from Bristol-Myers Squibb (Denmark), Vincristine from Faulding (Denmark), Ara-C and doxorubicin from Pharmacia A/S (Denmark). TNFα was from Genentech, CA, USA.

2.3. Clonogenic assay

The clonogenic assay was performed as previously described [13]. Cells used in the clonogenic experiments were in passage 35 for Pwt-I fibrosarcoma cells and passage 50 for Pko-I fibrosarcoma cells. In brief, drugs and cells were mixed, and then a mixture of agar and media was added. Gentle aspiration was repeated using a 1-ml syringe to achieve single cell suspension. A 1-ml volume was then plated in triplicate in Petri dishes upon a feeder layer of sheep red blood cells. When the agar had solidified, 1-ml media was added on top. Cells were grown in a CO_2 incubator (CO_2: 7.5%) at 37 °C and 100% humidified air. After three weeks pictures of the Petri dishes were taken and colonies (>64 cells) were counted with the use of the software Sorcerer (Perceptive Instruments, Suffolk, United Kingdom (UK)).

2.4. Cytotoxicity assay

Under cell culture conditions, cells that have been given an apoptotic stimulus will initially die by apoptosis, and later turn into secondary necrosis due to the lack of phagocytosis. Cytotoxicity or cell lysis can be measured by the release of lactate dehydrogenase (LDH) in the culture supernatant. For this purpose the 'Cytotoxicity Detection Kit' (Roche, Mannheim, Germany) was employed. Pko-I (passage 59–61) and Pwt-I fibrosarcoma cells (passage 62–64) were seeded in 96-well microtitre plate (2500 cells/well). After 24 h, cells were treated etoposide for 48 h, as indicated in Fig. 3(a) (below). A 50-μl volume of culture supernatant (total 200 μl) was transferred to a new 96-well microtitre plate and mixed with 50 μl of a substrate mix. The remaining supernatant was discarded, and the remaining intact cells were lysed by addition of 200 μl of lysis buffer (1% Triton-X100 in CM). Following lysis for 30 min at 37 °C, 50 μl of lysate was transferred to a new 96-well microtitre plate and mixed with 50 μl of a substrate mix. The cell culture supernatants and lysates were incubated for 10 min with the substrate mix protected from light. The absorbance was measured on a spectrophotometer at $\lambda_1 = 490$ nm and reference $\lambda_2 = 650$ nm. The amount of released LDH (%) was related to the total amount as follows:

$$\text{Cytotoxicity}(\%\text{LDH release}) = \frac{\text{LDH}_{\text{supernatant}}}{\text{Total LDH}(\text{LDH}_{\text{supernatant}} + \text{LDH}_{\text{lysate}})} \times 100\%.$$

2.5. Apoptosis assay

Apoptotic cell death can be measured by the presence of DNA–histone complexes in the cytoplasm. For this purpose the 'Cell Death Detection ELISA Kit' (Roche, Mannheim, Germany) was employed. Pko-I (passage 120) and Pwt-I fibrosarcoma cells (passage 119) were seeded in 96-well microtitre plate (5000 cells/well). After 24 h, cells were treated with etoposide for another 24 h, as indicated in Fig. 3(b) (below), and the level of apoptosis was measured with the 'Cell Death Detection ELISA Kit' according to the manufacturer's instructions.

2.6. Cell proliferation assay

Cell proliferation was estimated using the CyQuant® Cell Proliferation Assay Kit (Molecular Probes Inc., Eugene, OR, USA). 500 cells/well were used and the DNA content was measured at 480 nm excitation and 520 nm emission. Cell proliferation was measured daily over a period of 5 d. Pwt-I was in passage 60 and Pko-I was in passage 57 when the doubling times were calculated.

2.7. Western blotting

Cells were seeded in Petri dishes (4×10^5/dish). After 24 h the medium was changed to medium containing 0.5% FCS followed by 24 h incubation. The medium was harvested and floating cells were removed by centrifugation. Proteins were precipitated by adding 5% trichloroacetic acid (TCA; Riedel-de Haen, Germany) to the clarified media, followed by 10-min incubation on ice and centrifugation (10,000 g/10 min). The pellet was resuspended in buffer (25 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% triton; pH 7.5) and protein concentration was determined by protein BCA Protein assay kit (PIERCE, Rockford, IL, USA). Proteins (100 μg) were separated on an 8% polyacrylamide gel and blotted on nitrocellulose paper. The blot was blocked in phosphate-buffered saline (PBS) + 0.1 Tween 20 containing 5% dried milk for 1 h, and then incubated overnight with the primary antibody (polyclonal αPAI-1, a gift from Peter Andreasen, Aarhus University, Denmark): 3 μg/ml in PBS + 0.1 Tween 20 containing 1% dried milk. Subsequently, the blot was washed 3×10 min in PBS + 0.1% Tween 20, incubated with the horse peroxidase conjugated secondary antibody (1:1000 goat anti-rabbit antibody (DAKO, Denmark) in PBS + 0.1% Tween 20 containing 1% dried milk;) for 1 h followed by 3×10 min washing in PBS + 0.1% Tween 20. The blot was developed by the ECL + detection system (Amersham, UK) according to the manufacturer's instructions.

2.8. Animals and toxicity experiments in vivo

Generation of the PAI-1 gene-deficient mouse has been described previously [14]. The PAI-1 gene-targeted mouse was crossed into the META^{TM} /Bom-*nu* (=META^{TM}/Bom *nu/nu*) [15] athymic nude mouse and back-crossed for eight generations. The mice used are pairs of siblings representing homozygous gene-deficient and homozygous wild-type mice obtained by heterozygous breeding. Within an experiment involving wild-type mice as controls these were litter-mates to the PAI-1 deficient mice, and therefore, each separate experiment only included mice from the same back-crossed generation. All mice were obtained from Taconics M&B, Denmark, and were 10 weeks old at the beginning of the experiment. PAI-I +/+ male ($n = 4$) and female ($n = 6$) Meta nu/nu mice, as well as PAI-I −/− male ($n = 8$) and female ($n = 6$) mice were injected intraperitoneally with 75 mg/kg etoposide. PAI-I +/+ male ($n = 3$) and female ($n = 4$) mice, as well as PAI-I −/− male ($n = 5$) and female ($n = 4$) mice were injected intraperitoneally with vehicle control. The mice were kept in isolation on a 12-h day/night cycle and were fed regularly. Effect measures were weight loss and white blood cell count (WBC). The mice were weighed daily for a week. Blood (20 μl) was sampled by tail vein incision on days 3 and 5, and evaluated haematologically by automatic counting in a CA530-VET (Boule Medical AB, Sweden). All experiments were performed according to the guidelines published by the Danish Animal Experiments Inspectorate.

2.9. Statistics

The SAS® software package (version 8.2; SAS Institute, Cary, NC, USA) was used for statistical analysis. Repeated measurements of WBCs and weight were log transformed. The analysis fluctuations over time were carried out using a generalised linear model assuming a normal distribution and taking repeated measures into account. Estimates were obtained using generalised estimating equations [16]. *P*-values less than 5% were considered significant.

3. Results

PAI-1 has been shown to protect cancer cells from apoptosis when recombinant PAI-1 was added to the cell culture [9]. To study further this new function of PAI-1, PAI-1 gene-deficient and wild-type fibrosarcoma cell lines were established. When the Pko-I and Pwt-I cells were tested for tumourgenicity in mice at passage

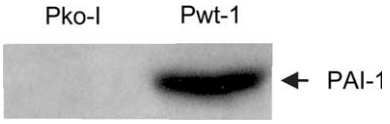

Fig. 1. Plasminogen activator inhibitor-1 (PAI-1) is secreted from Pwt-I fibrosarcoma cells. Condition media from Pko-I and Pwt-I fibrosarcoma cells were analysed by Western blotting using an anti-PAI-1 antibody.

28 and 34, respectively, it was found that both cell lines formed progressively growing tumours (data not shown). Furthermore, the cell lines formed colonies in soft agar (see below), were anaploid and demonstrated immortalised growth (all cell lines > passage 30), all of which supports that spontaneous malignant transformations have taken place. To verify that PAI-1 was expressed in Pwt-I cells and not in Pko-I cells RT-PCR and immunoblotting was performed. Fig. 1 shows that PAI-1 is expressed only in the Pwt-I fibrosarcoma cell line and that significant amounts of PAI-1 protein from these cells are secreted into the media. Next, the sensitivity of PAI-1 wild-type and PAI-1 gene-deficient fibrosarcoma cells to treatment with etoposide, doxorubicin, vincristine and Ara-C, was tested in a clonogenic assay. The cells were exposed to the drug-containing media and subsequently embedded in soft agar for three weeks. Clones (containing more than 64 cells) derived from the surviving cells were then counted. The advantage of the clonogenic compared with other cytotoxicity assays is that it is possible to treat the cells with low doses of the drugs for a longer period of time. The results in Figs. 2(a)–(d) shows that Pko-I fibrosarcoma cells form significantly fewer clones than Pwt-I fibrosarcoma cells when the cells were treated with each of the four chemotherapeutic agents. This suggests that PAI-1 gene-deficiency renders fibrosarcoma cell sensitive to apoptosis. Since PAI-1 expression might influence the rate of growth and thereby sensitivity to the chemotherapeutic drugs applied, the cell doubling times of Pwt-1 and Pko-1 were investigated. Using a cell proliferation assay (CyQuant®), it was found that the two cell lines had similar proliferation rates (Pwt-1: 0.98 d; Pko-1: 0.85 d). To verify that the decrease in surviving Pko-I clones was a result of an increase in cell death, the cytotoxicity to treatment with etoposide was analysed. Treatment with etoposide for 48 h induced dose-dependent cytotoxicity of both Pwt-I and Pko-I fibrosarcoma cells (Fig. 3(a)) determined by LDH release. However, Pko-I fibrosarcoma cells were significant more sensitive than Pwt-I fibrosarcoma cells to etoposide-induced cytotoxicity at all concentrations. To confirm that the cell death induced by etoposide was apoptotic, the level of cytoplasmic DNA–histone complexes in response to etoposide treatment was analysed. Indeed, Pko-I fibrosarcoma cells were significantly more sensitive to apoptosis than Pwt-I fibrosarcoma cells (Fig. 3(b)). The increased sensi-

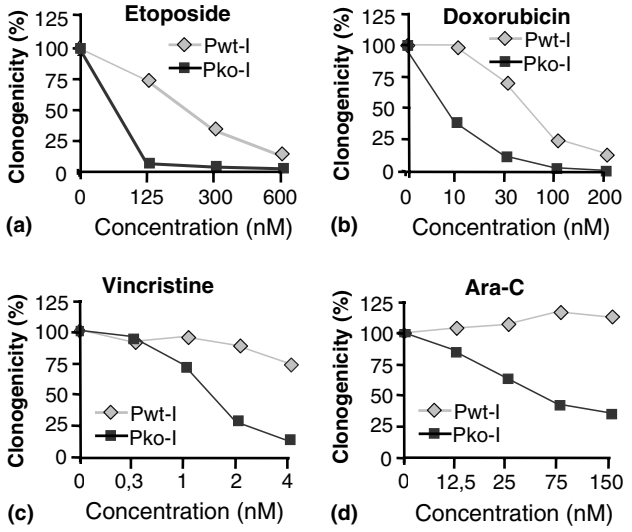

Fig. 2. Pko-I fibrosarcoma cells form significantly fewer clones than Pwt-I fibrosarcoma cells when treated with chemotherapeutic drugs. (a)–(d) Pko-I and Pwt-I fibrosarcoma cells were plated in soft agar containing chemotherapeutic drugs. The clonogenic potential was determined after three weeks of incubation by counting clones containing more then 64 cells. The clonogenicity was calculated as number of clones in drug-containing agar relative to number of clones in control-agar. The values represent means of a triplet determination ± SD.

tivity of Pko-I fibrosarcoma cells to treatment with etoposide were repeated and confirmed in the second pair of PAI-1 wild-type and gene-deficient fibrosarcoma cell lines, passage 34–36 (data not shown).

Next, we asked the question whether the difference in sensitivity also could be observed in the early passages of the Pko-I and Pwt-I cells. The cells were split and analysed for sensitivity to etoposide every week for 10 passages. Moreover, conditioned media were harvested for each passage to be analysed for the secretion of PAI-1. As illustrated in Fig. 4, Pko-I and Pwt-I cells display equal sensitivity to etoposide (~50% cytotoxicity) until passage 9. However, after passage 9 Pwt-I cells were protected from apoptosis (~20% cytotoxicity) whereas Pko-I cells displayed unchanged sensitivity to treatment with etoposide until passage 13 when a slight increase in sensitivity was observed (Fig. 4). For both cell lines the sensitivity to apoptosis was unchanged in subsequent passages.

The change in sensitivity to apoptosis between passages 9 and 10 could indicate a genetic alteration. Although, the Pwt-I cells were anaploid at all the measured passages (5–12), no major genetic alterations were observed during these passages (data not shown). This, on the other hand, does not role out that a mutation had occurred. It was also analysed whether the secretion level of PAI-1 changed between passages 9 and 10; however the expression level of PAI-1 remained stable at all passages (data not shown). Altogether, the fact that wild-type and PAI-1 gene-deficient cells display equal

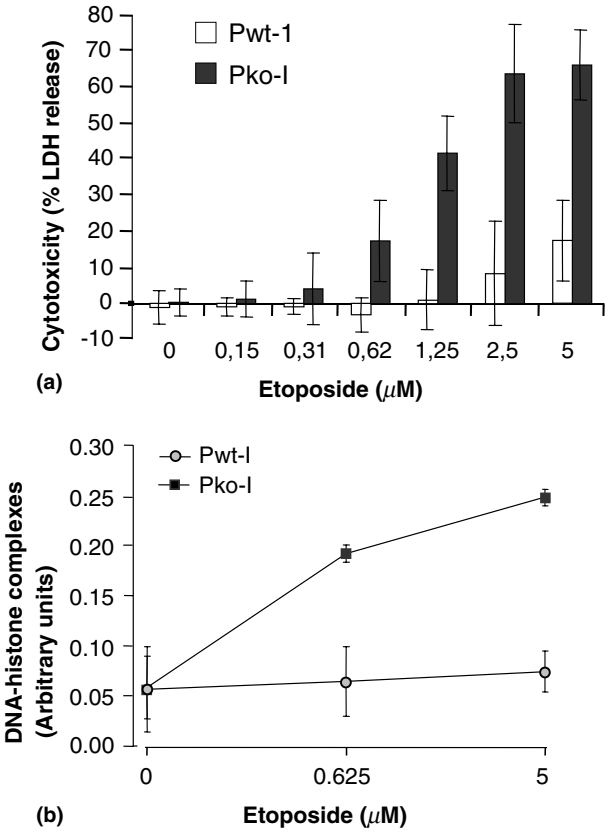

Fig. 3. Pwt-I fibrosarcoma cells are significantly less sensitive than Pko-I fibrosarcoma cells to apoptosis induced by etoposide. (a) Pko-I and Pwt-I fibrosarcoma cells were treated with etoposide for 48 h and cytotoxicity was measured as released lactate dehydrogenase (LDH) activity (% of total activity). Values represent means of three independent experiments ±SD. (b) Pko-I and Pwt-I fibrosarcoma cells were treated with etoposide for 24 h and DNA–histone complexes in the cytoplasm were detected using a 'Cell Death Detection ELISA Kit'. Values represent means of triplet determination ± SD and the experiment was performed more than three times with the same results.

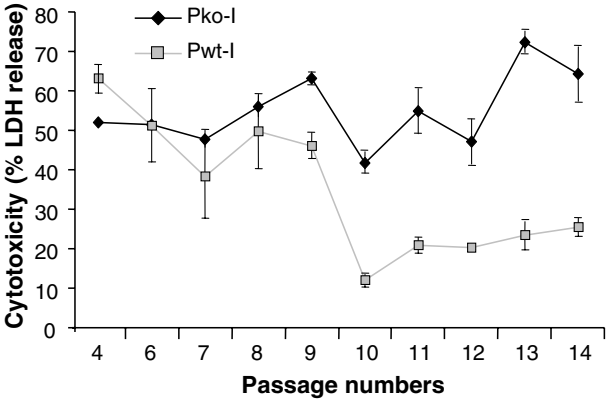

Fig. 4. Pwt-1 cells become protected from etoposide-induced apoptosis after passage 9. At each passage Pko-I and Pwt-I cells were treated with etoposide (5 μM) for 48 h and cytotoxicity was measured as released lactate dehydrogenase (LDH) activity (% of total activity). Values represent means of triplet determination ± SD and the experiment was repeated once with the same result.

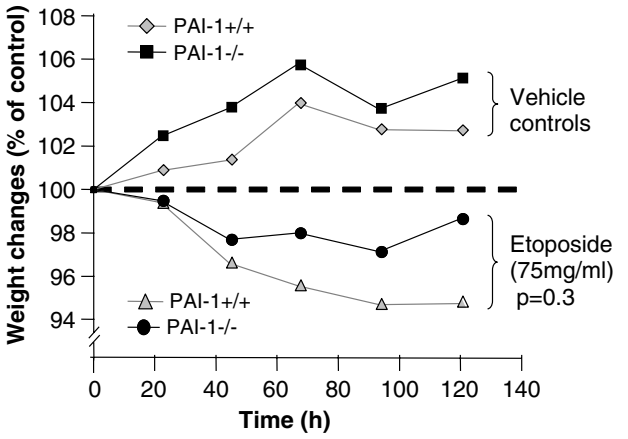

Fig. 5. Wild-type and PAI-1 gene-deficient mice display equal sensitivity to treatment with 75 mg/kg etoposide. Weight changes in Meta nu/nu PAI-1 gene-deficient and wild-type mice after treatment with 75 mg/kg etoposide or vehicle control compared with day of treatment (=100%).

sensitivity to apoptosis during the early passages and only wild-type transformed cells become protected from apoptosis during later passages suggests that PAI-1 is a regulator of apoptosis in malignant cells.

Since wild-type and PAI-1 gene-deficient cells display equal sensitivity to etoposide-induced apoptosis at the early passages is it possible that non-transformed wild-type and PAI-1 gene-deficient cells also are equally sensitivity to etoposide-induced apoptosis. To study this possibility, wild-type and PAI-1 gene-deficient mice were treated with etoposide. By statistical normalisation to initial weight, the effect of treatment was a weight loss for the treated mice compared with the untreated ($P < 0.0001$), while no difference was seen between genotypes ($P = 0.30$) or gender ($P = 0.41$) (Fig. 5). Etoposide suppresses WBC in both genotypes with a nadir on day 3 ($P = 0.0003$), but there was no difference ($P = 0.99$) in the response between wild-type and PAI-1 gene-deficient mice, or between males or females ($P = 0.58$) (data not shown). This preliminary assessment of weight loss and haematological toxicities was evaluated using a dose of etoposide close to LD_{10} for this compound. A more detailed toxicological profile of etoposide in the two genotypes could, if needed, be further evaluated.

4. Discussion

These results suggest a differential sensitivity between cancer cells and normal cells to apoptosis inhibition by PAI-1. This differential sensitivity makes PAI-1 an attractive target in combination with chemotherapeutic drugs, that is, the patient could be treated with a PAI-1 inhibitor to sensitise cancer cells to subsequent chemotherapy with no additional toxicity in normal tissue.

Little is known about how PAI-1 regulates apoptosis. It has been reported that addition of recombinant PAI-1 to HL-60 and PC3 tumour cells inhibits apoptosis induced by camptothecin or etoposide [9], suggesting that PAI-1 regulates apoptosis extracellularly. It was also demonstrated that the plasminogen activation system or binding to vitronectin was not involved in the inhibitory effect induced by PAI-1, suggesting that PAI-1 interacts *via* an unidentified receptor/pathway. It is also possible that it is intracellular PAI-1 rather than extracellular PAI-1 that is important for the inhibition of apoptosis. In support of this assumption, intracellular rather than extracellular PAI-2 has been shown to inhibit TNFα-induced apoptosis [17,18]. Furthermore, it has been suggested that PAI-1 might inhibit apoptosis of vascular smooth muscle cells by directly interacting with the intracellular death protease, caspase-3 [19]. Studies on how PAI-1 regulates apoptosis are ongoing.

In summary, one explanation for the observation that high levels of PAI-1 contribute to tumour progression is, in part, that PAI-1 inhibits apoptosis of tumour cells. This study provides data that suggests a differential sensitivity between normal and cancer cells to the inhibition of apoptosis by PAI-1. Development of compounds that abolish the anti-apoptotic function of PAI-1 might be useful in combination with conventional chemotherapeutic drugs in the treatment of cancer.

Conflict of interest statement

None declared.

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