

Plasminogen Activator Inhibitor 1 Protects Fibrosarcoma Cells from Etoposide-Induced Apoptosis through Activation of the PI3K/Akt Cell Survival Pathway¹

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Abstract

High levels of plasminogen activator inhibitor (PAI-1) in tumors are associated with poor prognosis in several cancer types, and the reason for this association is not fully understood. Plasminogen activator inhibitor 1 has been suggested to contribute to tumor growth by protecting cancer cells from apoptosis, and we have previously shown that wild type murine fibrosarcoma cells are significantly more resistant to apoptosis induced by chemotherapy than PAI-1-deficient fibrosarcoma cells. Here, we further investigated the molecular mechanisms underlying the antiapoptotic function of PAI-1 focusing on the phosphatidylinositol 3-phosphate kinase (PI3K)/Akt cell survival pathway. We demonstrate that the activation level of the Akt cell survival pathway is reduced in PAI-1-deficient cells. Inhibition of either PI3K or Akt by synthetic inhibitors sensitized the wild type but not the PAI-1-deficient cells to etoposide-induced cell death. More importantly, reintroduction of PAI-1 expression in PAI-1-deficient cells induced an increase in Akt activity and protection against etoposide-induced apoptosis. Concordantly, silencing of PAI-1 by RNA interference in wild type fibrosarcoma cells decreased the level of active Akt, and this was accompanied by a sensitization of the cells to etoposide-induced cell death. Altogether, our data suggest that PAI-1 influences sensitivity to etoposide-induced apoptosis through the PI3K/Akt cell survival pathway by acting upstream of PI3K and Akt. This points to PAI-1 as a possible therapeutic target in cancer diseases where PAI-1 inhibits chemotherapy-induced apoptosis.

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Introduction

The plasminogen activator system plays an important role in tumor growth and metastasis [1]. Urokinase-type plasminogen activator (uPA) is an activator of plasminogen and has been reported to have several tumor-promoting effects [2–4]. A large number of clinical studies have shown a strong association between high tumor tissue levels of uPA and poor cancer patient survival, supporting a major role of uPA in cancer progression. The plasminogen activator system is regulated by endogenous inhibitors, the serpin (serine protease inhibitor) gene family. One of these, plasminogen activator inhibitor 1 (PAI-1), is a secreted protein and is a major inhibitor of uPA [1]. On the basis of the many preclinical and clinical studies that link uPA to tumor progression, high PAI-1 levels in a tumor would be expected to inhibit cancer progression in patients and thus relate to a more favorable prognosis. However, a very large number of clinical studies

have demonstrated that high PAI-1 levels in tumors are associated with poor prognosis in cancer [5–8]. The results have now reach a

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level of evidence that have prompted the American Association for Clinical Oncology (ASCO) to recommend uPA and PAI-1 as prognostic variables in patients with breast cancer [9].

Several mechanisms have been proposed to explain this apparent discrepancy between what might be expected from high levels of PAI-1 and the observed clinical correlations. Plasminogen activator inhibitor 1 has been shown to stimulate angiogenesis [10–12], to mediate/stimulate cell migration [13,14], and to modulate cell adhesion [15]. It has also been suggested that PAI-1 modulates cell signaling, and when it is bound to uPA, it can promote growth of MCF-7 breast cancer cells through a sustained phosphorylation of the extracellular signal-regulated kinases 1/2 (Erk1/2) [16]. Furthermore, PAI-1 has been suggested to inhibit apoptosis in vascular smooth muscle cells through direct interaction with caspase-3 [17]. Finally, incubation of cancer cell lines with recombinant PAI-1 has been demonstrated to inhibit apoptosis induced by cytotoxic drugs [18]. It is still not known how PAI-1 mediates an antiapoptotic signal from the extracellular space into the cell and which signaling pathways might be involved.

One pathway involved in a variety of cellular responses, including the transmission of antiapoptotic survival signals leading to drug resistance, is the phosphatidylinositol 3-kinase (PI3K)/Akt cell signaling pathway [19,20]. The three Akt family members identified in mammals — Akt1/PKB α , Akt2/PKB β , and Akt3/PKB δ [21] — are activated by various stimuli in a PI3K-dependent manner [22,23] and depend on phosphorylation of Thr308 and Ser473 [24]. Activated Akt phosphorylates a number of target proteins involved in various cellular functions. For instance, the proapoptotic protein Bad is phosphorylated to promote cell survival and phosphorylation of Raf1 leads to inhibition of the Raf-MEK-ERK signaling pathway [25]. Furthermore, Akt phosphorylates and inactivates glycogen synthase kinase-3 β (GSK-3 β), leading to inhibition of glycogen synthesis [26]. The tumor-suppressor phosphatase and tensin homolog (PTEN), which is deleted or inactivated in many tumors, antagonizes the PI3K/Akt cell signaling pathway [27]. Loss of PTEN expression is known to cause Akt hyperactivation, leading to protection from various apoptotic stimuli [28,29]. It has recently been demonstrated that PAI-1 can regulate Akt activity that may involve inactivation of PTEN [30]. We have previously shown that malignantly transformed PAI-1-deficient cells are significantly more sensitive to apoptosis induced by cytotoxic drugs than are wild type cells [31].

The aim of the present study was to investigate whether the PI3K/Akt signaling pathway is involved in PAI-1-mediated inhibition of drug-induced programmed cell death in murine fibrosarcoma cells. The study describes the effect of selective inhibition of either PI3K or Akt on chemotherapy-induced apoptosis in PAI-1 expressing wild type and PAI-1-deficient murine fibrosarcoma cells. In addition, we describe the effect of specific silencing of PAI-1 in wild type cells on Akt activation and chemotherapy-induced apoptosis.

Materials and Methods

Cell Culture

Murine fibrosarcoma cells derived from either PAI-1-deficient or wild type mice were generated as previously described [32]. In brief, mice were bred heterozygous to heterozygous to obtain relevant pairs of siblings representing gene-deficient and homozygous wild type mice. Two pairs of fibrosarcoma cell lines were established,

and each pair of cell lines originated from mice born in the same litter: Pko-I (PAI-1 gene-deficient), Pwt-I (wild type) and Pko-II (PAI-1-deficient), Pwt-II (wild type). The cells were cultured in M199/Hanks salts (GIBCO, Invitrogen A/S, Taastrup, Denmark) supplemented with 10% fetal calf serum (complete medium, CM) at 37°C and 5% CO₂.

Compounds

Ly294002, Akt inh. VIII, and human insulin-like growth factor (IGF-1) were from Calbiochem (San Diego, CA). Etoposide was from Bristol-Myers Squibb (Lyngby, Denmark). Recombinant human PAI-1 and recombinant murine PAI-1 purified from *Escherichia coli* were from American Diagnostica, Inc., Stamford, CT.

Regulation of Akt Activity

The cell lines were analyzed for the capacity to regulate the PI3K/Akt signaling pathway by treating the cells with IGF-1 and etoposide. Cells were seeded in 10-cm² Petri dishes (4 × 10⁵ cells per dish) and grown for 24 hours before treatment. Cells were left untreated or treated with 100 ng/ml IGF-1 for 15 minutes or 10 μ M etoposide for 4 and 24 hours. IGF-1-treated and -untreated cells were harvested, and proteins were separated by SDS-PAGE and phosphor-Akt levels were detected according to the immunoblot analysis protocol (see below). Etoposide-treated and -untreated cells were harvested in cell lysis buffer without phosphatase inhibitors, and Akt activity was measured according to the Akt kinase assay kit (see below).

RNA Interference

Cells were seeded in 75-cm² cell culture flask in CM without antibiotics. When cells were approximately 30% to 50% confluent, the cells were transfected with Stealth RNAi (Invitrogen A/S) using XtremeGENE siRNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

Two different RNAi constructs directed to PAI-1 mRNA were used (PAI-1 RNAi), one attaching to the transcript at 375 in exon 2, the other at 899 in exon 5. The targeted sequences were as follows: UGACUUUGAAUCCCAUAGCAUCUUG (#2) and AAAGG-GUGCAGCGAUGAACAUUGCUG (#9).

As controls for the specificity of the PAI-1 silencing RNAi constructs, two corresponding mismatch constructs were used (Ctl RNAi): UGAUCUCGUUUAACCCAUACGUUG (#2C) and AAAGUG-GUGGCGACGUAGACAACUG (#9C). Cells were treated for 4 to 144 hours with PAI-1 RNAi constructs at concentrations of 35 to 64 nM, as optimal for silencing of PAI-1 with minimal cytotoxicity.

Cytotoxicity Assay

For measurement of cytotoxicity, the lactate dehydrogenase (LDH) release assay (Roche Applied Sciences, Mannheim, Germany) was used. Cells were treated with PAI-1 RNAi or Ctl RNAi for 51 to 78 hours, then trypsinized and seeded in a 96-well plate (3500 cells per well). After 24 hours, the cells were treated with indicated concentrations (Figures 2, C–D, and 5, A–B) of etoposide for 30 to 48 hours. LDH release was measured, and cytotoxicity was calculated as previously described [31].

Apoptosis Assay

Apoptotic cell death can be measured by the presence of oligonucleosomes in the cytoplasm. For this purpose, the Cell Death Detection ELISA Kit (Roche, Mannheim, Germany) was used. Cells were

seeded in a 96-well plate (3500 cells/well). After 24 hours, cells were treated with etoposide for another 48 hours, and the level of apoptosis was measured with the Cell Death Detection ELISA Kit according to the manufacturer's instructions.

Reintroduction of PAI-1 in Pko-1 Cells

Murine PAI-1 was a clone from the pcDNA-3.1-neo vector (generously provided by Ann Gils) to the pcDNA-3.1-hyg vector. Transfections were performed using FuGENE 6 transfection reagent (Roche, Mannheim, Germany). Pko-1 cells were seeded out in a six-well plate with 1×10^5 cells/well in 2-ml medium. After 24 hours, the medium was changed, and the cells were transfected with pcDNA-3.1-PAI-1 and empty vector (transfection mix: 6:1 ratio of FuGENE reagent (μ l) to plasmid DNA (μ g)). After 48 hours, medium was changed to a medium containing 150 ng/ml hygromycin (Sigma-Aldrich, Brøndby, Denmark). After 14 days, selection of transfected cells was completed. To obtain single-cell clones with a high expression of PAI-1, dilution cloning was performed. To avoid clonal heterogeneity, four single-cell clones of Pko-1-transfected cells expressing medium and high levels of PAI-1 were mixed in equal ratio (Pko-PAI-1-pool) and four vector control-transfected single-cell clones were mixed in an equal ratio (Pko-vector-pool) to obtain pool population of PAI-1-expressing Pko-1 cells and vector control-transfected Pko-1 cells.

Akt Kinase Assay

Akt kinase activity was measured by an Akt kinase assay kit (Cell Signaling Technology, Inc., Danvers, MA). Briefly, cells were seeded in 6-cm Petri dishes (4×10^5 per dish) and grown for 24 hours. Medium was aspirated, and cells were washed twice in 500 μ l of ice-cold PBS. Cells were lysed for 5 minutes in 500 μ l of cell lysis buffer containing 1 mM Pefa-block. Then cells were scraped off, transferred to an Eppendorf tube, and sonicated on ice. Twenty microliters of immobilized anti-Akt antibody bead slurry was added to 400 μ l of cell lysate and incubated with gentle rocking overnight at 4°C. Cell lysate/immobilized antibody was centrifuged at 14,000g for 30 seconds at 4°C. Pellet was washed twice with 500 μ l of ice-cold cell lysis buffer and twice with 500 μ l of ice-cold kinase buffer. The pellet was suspended in 50 μ l of 1 \times kinase buffer supplemented with 1 μ l of 10 mM ATP and 1 μ g of GSK-3 fusion protein. The mixture was incubated for 30 minutes at 30°C. The reaction was terminated with 25 μ l 3 \times LSB + β ME Buffer and boiled for 5 minutes. Phosphorylated GSK-3 fusion protein was detected by SDS-PAGE with an anti-phospho-GSK-3 antibody (1:1000).

RNA Extraction and Reverse Transcription

Cells transfected with 50 nM of the two PAI-1 RNAi constructs, Ctl RNAi constructs or transfection reagent alone, were grown in Petri dishes at the indicated periods (Figure 3B). Cells were washed with cold PBS and then harvested by scraping with a sterile cell scraper and collected in Eppendorf tubes. The cells were washed again with cold PBS and centrifuged at 300g for 5 minutes at 4°C. The cell pellet was lysed in 175 μ l of lysis buffer (SV Total RNA Isolation System; Promega, Madison, WI) and stored at -80°C until RNA extraction. When all samples were collected, RNA was extracted with a spin column kit (SV Total RNA Isolation System; Promega) according to the manufacturer's instructions. This procedure includes an on-column DNase treatment, minimizing the risk

of DNA contamination. The concentration of total RNA was measured spectrophotometrically.

Two micrograms of total RNA were transcribed into cDNA using the first-strand cDNA Synthesis Kit (Fermentas, Helsingborg, Sweden). The total volume of the reaction was 25 μ l consisting of 1 \times reaction buffer, 0.8 mM dNTPs, 20 U of RiboLock RNase inhibitor, 0.5 μ g of oligo(dT) primer, 0.2 μ g of random hexamer primer, and 40 U of M-MuLV reverse transcriptase. Samples were incubated at 25°C for 10 minutes, followed by 42°C for 1 hour. The reaction was terminated by incubating at 95°C for 5 minutes followed by cooling on ice.

Quantitative Polymerase Chain Reaction

All primer sets used were intron spanning to avoid false-positive results from contaminating genomic DNA. All samples were measured in duplicate and β -actin was used as reference gene.

Primer sequences:

PAI-1 forward: 3'-ACGTTGTGGAAGCTGCCCTAC-5';

PAI-1 reverse: 3'-GCCAGGGTTGCACTAAACAT-5'.

β -actin forward: 3'-CGTGGGCCGCCCTAGGCACCA-5';

β -actin reverse: 3'-TTGGCCTTAGGGTTTCAGGGGG-5'.

Quantitative polymerase chain reaction (Q-PCR) was carried out using SYBR Green I detection and the LightCycler 480 (Roche Diagnostics, Hvidovre, Denmark). Reactions were carried out in 96-well plates with 2 μ l of cDNA in 20- μ l reaction volumes consisting of 1 \times FastStart Master SYBR Green Mix and 0.5 μ M of the gene-specific primer. The amplification program was as follows: preincubation for fast start polymerase activation at 95°C for 5 minutes, followed by 45 amplification cycles [95°C for 10 seconds (20°C/sec), 60°C for 10 seconds (20°C/sec), and 72°C for 10 to 12 seconds (20°C/sec)]. SYBR Green fluorescence was acquired at 72°C in each amplification cycle. After the end of the last cycle, the melting curve was generated by starting the fluorescence acquisition at 65°C and taking measurements every 0.1 seconds until 95°C was reached.

Relative quantification was done using the Relative Quantification software (LightCycler, Roche, Hvidovre, Denmark).

Immunoblot Analysis

Cells were seeded in 10-cm Petri dishes in CM. At 90% confluence, cells were washed with cold PBS, scraped off the culture dish with a sterile cell scraper, and collected in Eppendorf tubes. The cell pellet was resuspended in 50 μ l of lysis buffer (0.5% Triton X-100, 25 mM HEPES, 1.5 mM MgCl₂, 1 mM EGTA) supplemented with protease inhibitors (10 μ g/ml aprotinin, 1 μ g/ml pepstatin a, 1 μ g/ml leupeptin, 1 μ M Pefa-block) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate). All inhibitors were from Sigma-Aldrich Denmark A/S.

Cells were left on ice for 30 minutes followed by centrifugation at 20,000 g for 5 minutes at 4°C. The protein concentration in each sample was quantified by BCA Protein Assay Kit (Pierce, Rockford, IL). Cell lysates were mixed with 0.25 volume 4 \times Laemmli sample buffer containing 5% β -mercaptoethanol and boiled for 5 minutes. Equal amounts of protein were separated by SDS-gel electrophoresis using a 12% polyacrylamide gel and blotted on nitrocellulose paper. The blot was blocked in PBS + 0.1% Tween 20 containing 5% dry milk for 1 hour.

The primary antibodies used were anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Raf1 (Ser259), anti-Raf1, anti-phospho-GSK-3 β (Ser9), anti-GSK-3 β , anti-phospho-PTEN (Ser380), anti-PTEN (all diluted 1:1000; Cell Signaling Technology Inc.), anti-murine PAI-1 (3 μ g/ml; American Diagnostica, Inc.), and GAPDH (1:80,000; Biogenesis, UK). The blots were incubated with primary antibodies, diluted in PBS, supplemented with 0.1 Tween 20 and 1% dry milk (5% dry milk for anti-PAI-1 antibody) over night, then washed 3 \times in PBS supplemented with 0.1 Tween 20, and subsequently incubated with respective HRP-conjugated secondary antibodies (1:2000 to 1:10,000; Dako, Glostrup, Denmark A/S) in PBS supplemented with 0.1% Tween 20 and 1% dry milk. The blots were developed by the ECL detection system (Amersham Bioscience, Little Chalfont, UK) according to the manufacturer's instructions. The band densities were quantified by the UVP VisionWorksLS Image Acquisition and Analysis Software.

Statistics

Student's *t* test was used in the experiment where PAI-1 was reintroduced into the PAI-1-deficient cells to calculate if the reintroduction of PAI-1 induced a protection from cell death. In the experiment where PAI-1 was silenced by RNAi, a one-sample *t* test was used to calculate if fold induction in cytotoxicity had a distribution different from 1. *P* values were considered significant when *P* < .05.

Results

Activation Level of PI3K/Akt Signaling Pathway Is Reduced in PAI-1-Deficient Fibrosarcoma Cells

First, the activation status of the PI3K/Akt cell signaling pathway in PAI-1-expressing and PAI-1-deficient fibrosarcoma cell lines were analyzed (Figure 1). The wild type cell lines (Pwt-I and Pwt-II) showed significantly higher levels of Akt phosphorylation than the PAI-1-deficient cell lines (Pko-I and Pko-II). In addition, the two downstream targets of Akt, Raf-1 and GSK-3 β , presented a higher degree of phosphorylation in wild type cells compared to levels in PAI-1-deficient cells, indicating that PAI-1 can activate the PI3K/Akt cell signaling pathway. Next, it was investigated if the expression levels or phosphorylation levels of the PI3K/Akt antagonist PTEN were different between PAI-1-deficient fibrosarcoma cells and wild type cells. No differences in either expression levels or phosphorylation levels of PTEN were observed (Figure 1). To analyze if PAI-1 can activate the Akt signaling pathway, recombinant human stable PAI-1 and recombinant murine PAI-1 (500 ng/ml, 24 hours) were added to PAI-1-deficient and PAI-1 wild type cells; however, no influence Akt phosphorylation level was observed (data not shown). This may be because the murine cells are not responsive to human PAI-1 and most of the murine PAI-1 is converted to its latent form before it can exert its effects on the cells. To analyze if the cell lines were responsive to external stimuli, wild type and PAI-1 gene-deficient cells were stimulated with IGF-1 and etoposide. Incubation with IGF-1 for 15 minutes induced a significant induction in Akt phosphorylation levels for both wild type and PAI-1-deficient cells (Figure 2A). Incubation with etoposide for 4 hours, however, resulted in a distinct reduction in Akt activity in Pko-I and Pwt-I cells (Figure 2B) and Pko-II and Pwt-II cells (data not shown). After 24 hours of treatment, Akt activity increased compared to the 4 hours of treatment in both Pko-1 and Pwt-I cells; however, the level of Akt

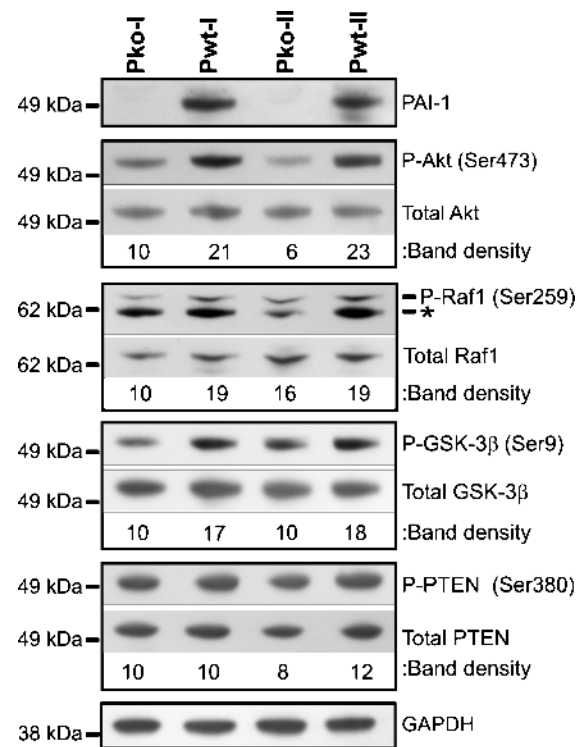


Figure 1. Akt is hyperactivated in wild type fibrosarcoma cells. One large preparation of cell lysates of each of Pko-I, Pwt-I, Pko-II, and Pwt-II cells, respectively, were used to detect PAI-1 and components of the Akt cell survival pathway. Proteins (50 μ g) were separated by SDS-PAGE, and the blots were probed with specific antibodies as indicated. After detecting phosphorylated forms of the proteins, the blots were stripped and reprobed with an antibody recognizing the total level of the protein. The anti-Akt and anti-phospho-Akt (P-Akt) antibodies recognize all three Akt family members. Band densities were calculated as the density of the phosphorylated protein divided by the density of the total amount of the protein. GAPDH served as a loading control. The asterisk indicates a background band. The immunoblot analysis was repeated once with a new preparation of cell lysate, and identical results were obtained.

activity was still lower than that of the untreated cells. These data demonstrate that the PI3K/Akt signaling pathways in both the wild type and PAI-1-deficient cell lines are responsive to growth promoter and growth inhibitor stimuli. Furthermore, a higher Akt activation level was observed in the Pwt-I cells compared to the Pko-I cells (Figure 2B), demonstrating that the level of phosphorylated Akt correlates with the level of Akt activity.

Wild Type Fibrosarcoma Cells Can Be Sensitized to Etoposide-Induced Cell Death by Inhibition of the PI3K/Akt Cell Signaling Pathway

To further analyze the role of the PI3K/AKT cell signaling pathway in PAI-1-mediated protection against apoptosis, the cells were incubated with specific synthetic inhibitors of PI3K and Akt followed by induction of cell death by etoposide. Incubation with the PI3K inhibitor Ly294002 or the Akt inhibitor Akt inh. VIII induced a dose-dependent inhibition of phosphorylated Akt in both wild type and PAI-1-deficient cells (Figure 3, A and B). To investigate if the observed inhibition of Akt phosphorylation levels affected the sensitivity to cell death of wild type and PAI-1-deficient cells, both



Figure 2. The PI3K/Akt signaling pathway is responsive to growth inducer and growth inhibitor in both wild type and PAI-1-deficient cells. Pko-I, Pko-II, Pwt-I, and Pwt-II cells were treated with IGF-1 for 15 minutes (A), and Pko-I and Pwt-I cell were treated with etoposide for 0, 4, and 24 hours (B). Phosphorylated Akt (P-Akt) was detected by immunoblot analysis with an antibody recognizing all three Akt family members phosphorylated on residue 473. Akt kinase activity was measured by incubating immunoprecipitated Akt from cell lysates with ATP and a GSK-3 fusion protein. The reaction was analyzed by immunoblot analysis, and phosphorylated GSK-3 fusion protein was detected by an anti-phospho-GSK-3 antibody. Band density was calculated as the density of the phosphorylated protein (B) or as the density of the phosphorylated protein divided by the density of the GAPDH (A). Band densities were normalized to the band density of the first lane(s) in both blots. GAPDH serves as a loading control. The experiments were repeated once with essentially same results.

cell lines were pretreated with Ly294002 or Akt inh. VIII followed by treatment with etoposide. Etoposide was chosen as death inducer because etoposide is used to treat sarcomas in the clinic. Treatment of wild type cells with etoposide together with Ly294002 or Akt inh. VIII induced an increase in cell death compared with cells treated with only etoposide (Figure 3C), whereas no increase in cell death was observed in PAI-1-deficient cells pretreated with the inhibitors (Figure 3D). The lack of induction in cytotoxicity by the inhibition of PI3K and Akt in the Pko-I cells may be because the Pko-I cells are highly sensitive to etoposide compared with wild type cells and, therefore, inhibition of the PI3K-Akt pathway will not further enhance the sensitivity of the cells. It was verified by the Cell Death Detection ELISA Kit (Roche) that the induction in cell death by pretreatment with PI3K and Akt inhibitors correlated with an increase in apoptosis (data not shown). Altogether, these results indicate that PAI-1 can protect wild type fibrosarcoma cells against apoptosis by activating the PI3K/Akt signaling survival pathway. It was further analyzed if treatment of wild type cells with Ly294002 or Akt inh. VIII induced PAI-1 expression; however, no induction in PAI-1 expression was observed (data not shown).

Reintroduction of PAI-1 in PAI-1-Deficient Cells Induces an Increased Akt Activation and Protection from Etoposide-Induced Apoptosis

To further investigate the involvement of the PI3K/Akt signaling pathway in PAI-1-mediated protection from etoposide-induced

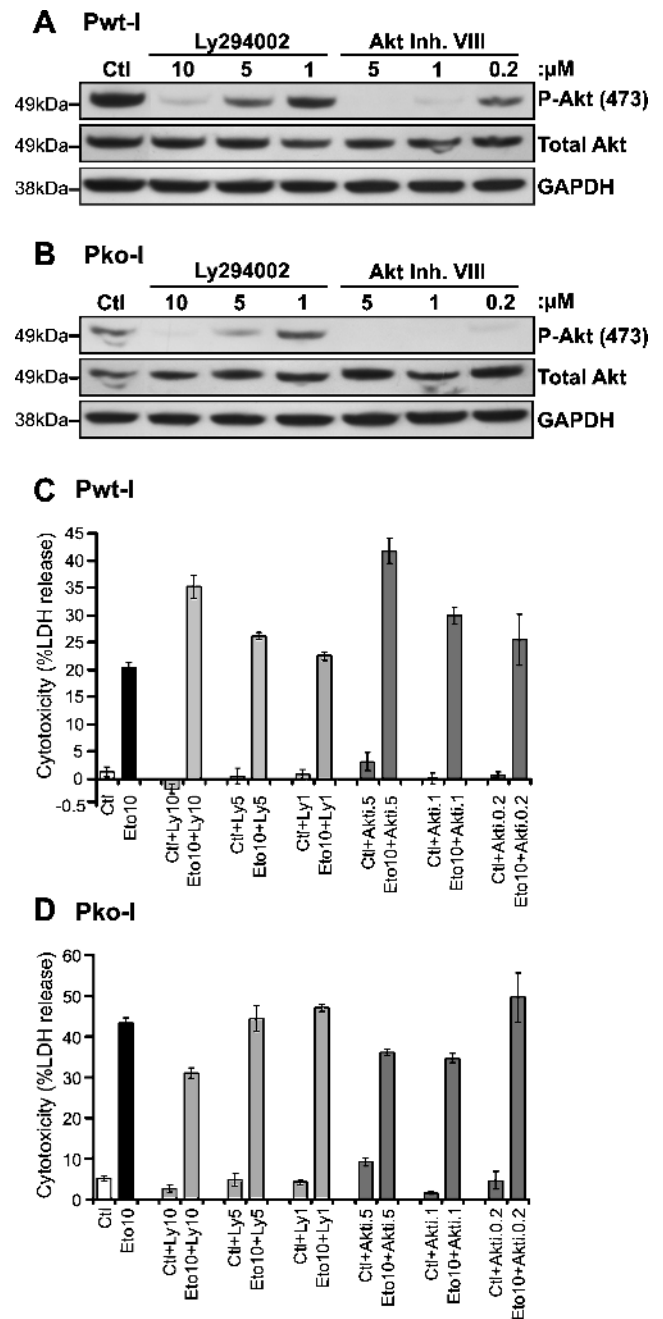


Figure 3. Inhibitors of the PI3K/Akt cell survival pathway sensitize wild type cells to etoposide-induced cell death. *Ctl.* indicates control; *Eto10*, etoposide 10 μ M; *Ly10*, 5, 1, Ly 294002 10, 5, 1 μ M; *Akt* 5, 1, 0.2, Akt inhibitor VIII 5, 1, 0.2 μ M. Pwt-I and Pko-I cells were treated with the inhibitor of PI3K (Ly294002) or Akt (Akt inhibitor VIII) for 2 hours (A, B) followed by 48 hours treatment with etoposide (C, D). Phosphorylated Akt (P-Akt) was detected by immunoblot analysis with an antibody recognizing all three Akt family members phosphorylated on residue 473, and total Akt was detected with a pan Akt antibody recognizing all three Akt family members. GAPDH served as a loading control. The immunoblot analysis was repeated once with identical result. Cell death was measured as percent LDH release. The cell death data shown is representative of four independent experiments showing the essential same result. Furthermore, the experiment was repeated with Pko-II and Pwt-II cells with essentially the same result.

apoptosis, we reintroduced PAI-1 into the PAI-1-deficient cells by stable transfection. Four single-cell clones expressing PAI-1 were pooled in equal ratio to make a pool population (Pko-PAI-1-pool), and PAI-1 expression from this pool population was lower but close to the expression level in wild type cells (Figure 4A). Interestingly, the Pko-PAI-1-pool cells had a higher Akt activation level compared to a pool population of four vector control single-cell clones (Pko-vector-pool; Figure 4B). To analyze if the reintroduction of PAI-1 affected the sensitivity of the cells to etoposide-induced cell death, etoposide-induced cytotoxicity and apoptosis of Pko-PAI-1-pool and Pko-vector-pool cells were measured (Figure 4, C and D). Pko-PAI-1-pool cells were significantly less sensitive to etoposide-induced cytotoxicity and apoptosis compared to Pko-vector-pool cells, indicating that

PAI-1 expression confers protection to the Pko-I cells. Altogether, these data further indicate that PAI-1 can protect fibrosarcoma cells from etoposide-induced apoptosis through activation of the PI3K/Akt signaling pathway.

RNAi-Mediated Silencing of PAI-1 Leads to a Reduction in Phosphorylated Akt

To establish a more direct link between PAI-1 expression and Akt activation, we used RNAi to specifically down-regulate PAI-1 and reassess the influence that PAI-1 has on the level of Akt phosphorylation. Transient transfection of wild type fibrosarcoma cells with RNAi directed against PAI-1 induced a reduction in PAI-1 protein levels, reaching its lowest levels between 48 and 95 hours after treatment (Figure 5A). The initial, significant increase in PAI-1 mRNA levels in both RNAi-treated cells and control-treated cells (Figure 5B) was found to be caused by addition of fresh CM to all cells when RNAi treatment was initiated. After RNAi treatment, PAI-1 mRNA levels gradually decreased, and PAI-1 mRNA levels in cells treated with PAI-1 RNAi showed a faster decrease and reached a lower level compared with PAI-1 mRNA levels in cells treated with the mismatching control RNAi construct. When corresponding PAI-1-protein levels were measured by immunoblot analysis, a perfect correlation between mRNA and protein levels was observed (Figure 5, A and B). Thus, treatment of wild type cells with RNAi directed against PAI-1 induced a specific silencing of PAI-1 expression.

RNAi-mediated silencing of PAI-1 in wild type fibrosarcoma cells resulted in a significant reduction of Akt phosphorylation level (Figure 6). The level of Akt phosphorylation was lower in wild type cells treated with PAI-1 RNAi construct compared with wild type cells treated with the transfection agent alone and with wild type cells treated with the mismatching control RNAi construct and corresponded to the levels of PAI-1 protein (Figure 6).

RNAi-Mediated Silencing of PAI-1 Sensitizes Fibrosarcoma Cells to Etoposide-Induced Cell Death

Next, we investigated if silencing of PAI-1 could sensitize the cells to etoposide-induced programmed cell death. Wild type cells were transfected with RNAi against PAI-1 or a mismatching control before etoposide treatment. Transfection with the control RNAi construct induced a minor sensitizing effect on etoposide-induced cell death, whereas RNAi-mediated silencing of PAI-1 induced a pronounced

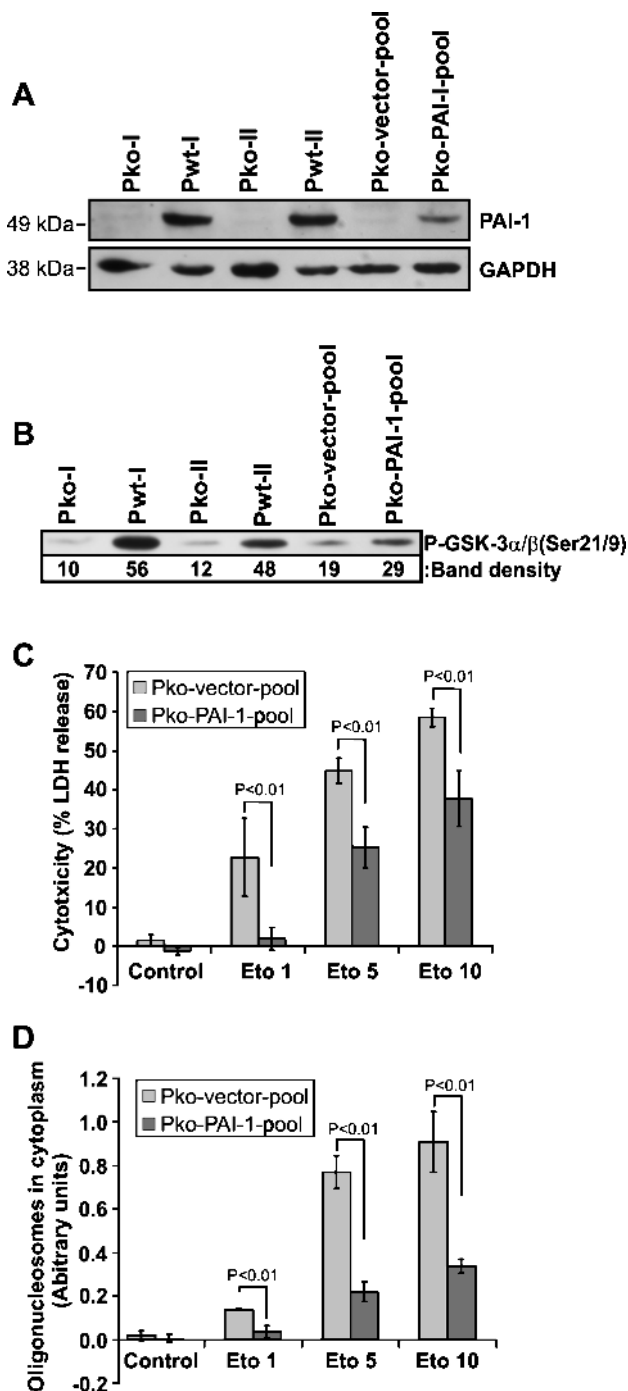


Figure 4. Reintroduction of PAI-1 in PAI-1-deficient cells induces an increased Akt activation and protection from etoposide-induced apoptosis. (A) PAI-1 expression levels in Pko-I, Pwt-I, Pko-II, Pwt-II, Pko-vector-pool, and Pko-PAI-1-pool were determined by immunoblot analysis with an anti-murine PAI-1 antibody (American Diagnostica, Inc.). GAPDH serves as loading control. (B) Akt kinase activity was measured by incubating immunoprecipitated Akt from cell lysates with ATP and a GSK-3 fusion protein. The reaction analyzed by immunoblot analysis and phosphorylated GSK-3 fusion protein was detected by an anti-phospho-GSK-3 antibody. Band densities were normalized to the band density of the first lane. The experiment was repeated at least once with essentially same result. (C, D) Pko-vector-pool and Pko-PAI-1 pool cells were grown in 24 hours before 48 hours of incubation with etoposide (μ M). Cytotoxicity was measured by LDH release (C) and apoptosis by oligonucleosomes in the cytoplasm (D). Results in (C) and (D) represent means of three independent experiments \pm SD.

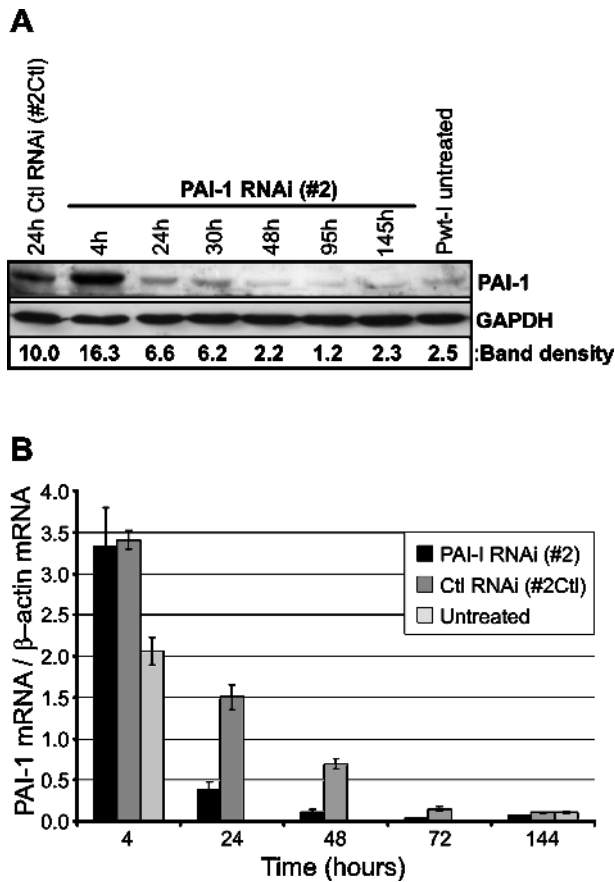


Figure 5. PAI-1 mRNA and protein is down-regulated by RNAi. (A) Pwt-I cells were treated with 50 nM PAI-1 RNAi (#2) or control RNAi (#2Ctl) or left untreated for the indicated periods. Equal amounts of proteins were analyzed for PAI-1 protein levels by immunoblot analysis. Lane 8: untreated Pwt-I cells at 95 hours. GAPDH served as a loading control. Band densities were normalized to the band density of the first lane. (B) PAI-1 mRNA levels in Pwt-I fibrosarcoma cells after treatment with 50 nM PAI-1 RNAi (#2) was quantified by Q-PCR. Plasminogen activator inhibitor 1 mRNA level was normalized to β -actin mRNA level. Detection of PAI-1 down-regulation by immunoblot analysis was performed twice with construct #2 and once with construct #9 with similar result and Q-PCR was performed once with each construct.

sensitizing effect (Figure 7A). The experiment was repeated with a second PAI-1 RNAi construct (#9) to ensure that this was a specific effect. The PAI-1 RNAi construct #9 induced identical sensitizing effect as PAI-1 RNAi construct #2, suggesting that the effect of PAI-1 silencing indeed is specific (Figure 7B; #9: $P < .01$ and #2: $P < .01$).

Discussion

In the present study, we show that the Akt cell signaling pathway is involved in PAI-1-mediated inhibition of etoposide-induced cell death. In unstimulated wild type fibrosarcoma cells, a higher Akt phosphorylation level was observed compared with PAI-1-deficient fibrosarcoma cells. Pretreatment of wild type fibrosarcoma cells with synthetic inhibitors of PI3K or Akt sensitized the cells to etoposide-induced cell death. A similar effect was not observed in PAI-1-deficient cells, indicating a role for the PI3K/Akt in PAI-1-expressing cells but not in PAI-1-deficient fibrosarcoma cells. In support of this as-

sumption, reintroduction of PAI-1 into the PAI-1-deficient cells resulted in an up-regulation of Akt activity and was accompanied by a decrease in sensitivity to etoposide-induced apoptosis. Furthermore, RNAi-mediated silencing of PAI-1 in wild type cells was accompanied with down-regulation of phosphorylated Akt. More importantly, silencing of PAI-1 by RNAi sensitized the cells to etoposide-induced cell death, demonstrating that PAI-1 is involved in protecting the cells against apoptosis. It is of interest to note that the levels of induction of cell death reached by the inhibition of PI3K and Akt before etoposide treatment were of the same magnitude as the induction gained by pretreatment of wild type cells with RNAi against PAI-1 before etoposide treatment. Thus, our data suggest that PAI-1 induces a survival signal through the Akt cell signaling pathway leading to resistance to etoposide-induced cell death.

Several lines of evidence suggest that PAI-1 expression can be induced by the PI3K/Akt signaling pathway. Plasminogen activator inhibitor 1 expression can be induced by nerve growth factor in rat pheochromocytoma cells (PC-12) and by hypoxia in hepatocellular carcinoma cells (Hep2G), and this induction can be inhibited by PI3K inhibitors, indicating that the PI3K/Akt signaling pathway positively regulates PAI-1 expression [33,34]. Conversely, overexpression of a dominant-negative mutant of PI3K or Akt increases TNF-alpha and insulin-induced PAI-1 expression in endothelial cells [35]. Likewise, silencing of Akt by RNAi enhances PAI-1 expression in ovarian cancer cells [36], suggesting that the PI3K/Akt cell signaling pathway negatively regulates PAI-1 expression in these cell types. However, when the PI3K/Akt cell signaling pathway was inhibited by synthetic inhibitors of PI3K and Akt, we did not observe an induction in PAI-1 expression in the wild type fibrosarcoma cells. Moreover, it was studied if addition of either human stable PAI-1 or recombinant murine PAI-1 to the cells induced the phosphorylation of Akt; however, no induction was observed. Contrary to our results, Balsara et al. [30] have shown that treatment of the PAI-1 gene-deficient endothelial cells with recombinant PAI-1 induced a decrease in phosphorylated Akt levels and increased apoptosis. Furthermore, Balsara et al. found that Akt is hyperactivated in PAI-1 gene-deficient primary endothelial cells compared with the level of activation in wild type primary

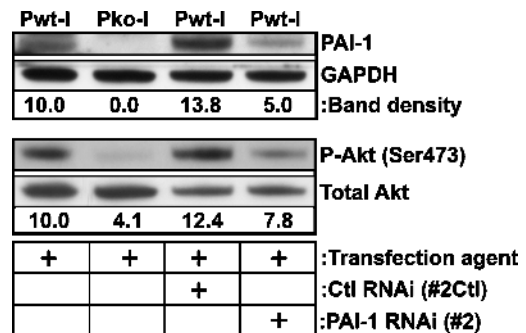


Figure 6. Silencing of PAI-1 results in a reduction in phosphorylated Akt. Equal amounts of proteins from Pko-I and Pwt-I fibrosarcoma cells treated for 120 hours with or without 50 nM RNAi (#2) were analyzed for protein levels of PAI-1, P-Akt (Ser473), and total-Akt by immunoblot analysis. GAPDH served as a loading control. Band densities of PAI-1 protein were normalized to band densities of GAPDH. Band densities of phospho-Akt protein were normalized to band densities of total Akt protein. The experiment was repeated once with RNAi construct #9 with similar result.

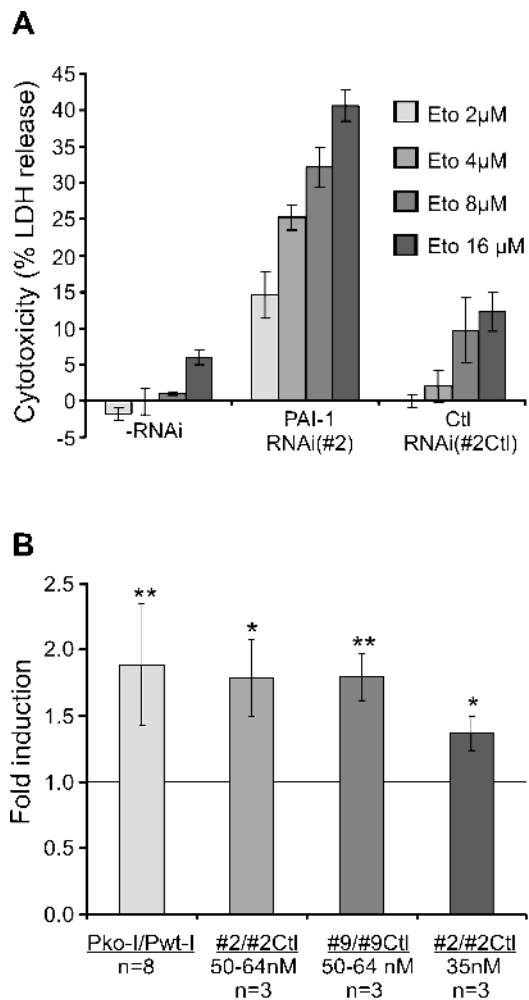


Figure 7. RNAi-mediated silencing of PAI-1 sensitizes fibrosarcoma cells to etoposide-induced cell death. (A) Pwt-1 and Pko-1 cells were treated with 64 nM RNAi (#2) against PAI-1 for 30 hours followed by treatment with etoposide for 48 hours. The cell death was measured as percent LDH release. (B) The fold induction in cytotoxicity was calculated as percent cell death of Pko-1 cells or PAI-1 RNAi-treated Pwt-1 cells divided with percent cell death of Pwt-1 cells or control RNAi-treated Pwt-1 cells, respectively. One sample *t* test was used to calculate *P* values by comparing the mean value to a hypothetical value of 1.00. Asterisks indicate: **P* < .05; ***P* < .01.

endothelial cells, and they suggested that this could be due to the inactivation of PTEN. The discrepancy between our data and those of Balsara et al. could rest in that PAI-1 induces different responses in primary cells compared with transformed cells. In support of this assumption, we have previously demonstrated that wild type and PAI-1 gene-deficient fibroblasts display equal sensitivity to etoposide-induced apoptosis in the early passages but wild type cells gain resistance to chemotherapeutic treatment during the immortalization/transformation process in the later passages [31].

Moreover, data by Kortlever et al. [37] suggest that PAI-1 is a critical downstream target of p53 in the induction of replicative senescence in primary fibroblasts and that PAI-1 knock-down leads to activation of the PI3K-Akt-GSK-3 β signaling pathway. They suggested that PAI-1 regulates replicative senescence by inhibiting the uPA-mediated increase in bioavailability of growth factors able to ac-

tivate the PI3K/Akt pathway. We have analyzed the levels of p53 in the fibroblast/fibrosarcoma cell lines and detected high p53 levels after passage 3 in the wild type cells and passage 6 in the PAI-1 gene-deficient cells, indicative of *P53* inactivation (Lademann U, unpublished results). In contrast to the studies by Kortlever et al., our studies were conducted with aneuploid fibrosarcoma cells in passage 30 (approximately 22 passages after crisis) [32]. It is likely to assume that the opposing results concerning the regulation of PI3K/Akt cell signaling pathway by PAI-1 and regulation of PAI-1 gene expression by Akt is related to the choice of cell model and cellular settings. This could also explain why we did not observe any effect on PAI-1 expression when inhibiting the PI3K/Akt cell signaling pathway in the wild type cell lines.

In summary, the present study provides new insight into how PAI-1 regulates programmed cell death in fibrosarcoma cells. The next challenge is to uncover if and by which mechanism the survival signal of PAI-1 is transmitted over the plasma membrane. Identification of the exact mechanism would enable the development of specific inhibitors of PAI-1 survival signaling providing a new way to overcome cancer cell resistance to chemotherapy.

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