Cell Cycle and Senescence

PAI-1 Leads to G1-Phase Cell-Cycle Progression through Cyclin D3/cdk4/6 Upregulation

Evan Gomes Giacoia1, Makito Miyake1, Adrienne Lawton3, Steve Goodison1,2,4, and Charles J. Rosser1,2

Abstract

The canonical function of plasminogen activator inhibitor-1 (PAI-1/SERPINE1) is as an inhibitor of urokinase-type plasminogen activator for blood clot maintenance, but it is now also considered a pleiotropic factor that can exert diverse cellular and tumorigenic effects. However, the mechanism controlling its pleiotropic effects is far from being understood. To elucidate the tumorigenic role of PAI-1, we tested the effects of PAI-1 after manipulation of its expression or through the use of a small-molecule inhibitor, tiplaxtinin. Downregulation of PAI-1 significantly reduced cellular proliferation through an inability to progress from the G0–G1 phase of the cell cycle. Accordingly, overexpression of PAI-1 augmented proliferation by encouraging S-phase entry. Biochemically, cell-cycle arrest was associated with the depletion of the G1-phase transition complexes, cyclin D3/cdk4/6 and cyclin E/cdk2, in parallel with the upregulation of the cell-cycle inhibitors p53, p21Cip1/Waf1, and p27Kip1. PAI-1 depletion significantly decreased the tumor size of urothelial T24 and UM-UC-14 xenografts, and overexpression of PAI-1 substantially increased the tumor size of HeLa xenografts. Finally, immunohistochemical analysis of human bladder and cervical tumor tissue microarrays revealed increased expression of PAI-1 in cancerous tissue, specifically in aggressive tumors, supporting the relevance of this molecule in human tumor biology.

Implications: Targeting PAI-1 has beneficial antitumoral effects and should be further investigated clinically. Mol Cancer Res; 12(3); 322–34. ©2014 AACR.

Introduction

PAI-1 is an endogenous inhibitor of urokinase-type plasminogen activator (uPA). Specifically, PAI-1 normally functions as part of the plasminogen activation system, which includes the serine protease uPA, its receptor uPA-R, tissue-type plasminogen activator (tPA), and inhibitors PAI-1 and PAI-2 (1–3). PAI-1 expression is regulated by many intrinsic factors (e.g., cytokines and growth factors) and extrinsic factors (e.g., cellular stress; ref. 4). Although the canonical function of PAI-1 has been known as an inhibitor of uPA to maintain clot formation, it is now regarded as a pleiotropic factor exerting diverse cellular effects, many potentially related to tumorigenesis, including cell migration, invasion, adhesion, and angiogenesis.

Some reports suggest that PAI-1 influences tumor progression indirectly, through effects on the extracellular matrix that perturb migration, invasion, and angiogenesis (5, 6), but other reports claim that PAI-1 itself promotes tumor growth directly (7). For example, high levels of PAI-1 in human and murine cancer cells were associated with the retardation of tumor growth and invasion in a rodent model (8, 9). Thus, the role of PAI-1 in tumorigenesis and growth is complicated, varying with experimental design and its cellular origin, so more preclinical and mechanistic studies are required to elucidate the role of PAI-1 in human cancers.

Disregulation of cellular proliferation is a hallmark of cancer (10–13). The cell cycle is a highly controlled process that involves tight regulation of key molecules that allow cells to progress between phases of the cell cycle. In an attempt to investigate the effect of PAI-1 on cell proliferation and cell-cycle progression, we created stable knockdown clones of PAI-1 in the urothelial T24 and UM-UC-14 cells and overexpressing PAI-1 clones in the cervical tumor cell HeLa. Next, we also inhibited PAI-1 activity with the small-molecule antagonist of PAI-1, tiplaxtinin (PAI-039; refs. 14, 15). Silencing of PAI-1 in T24 and UM-UC-14 cells via short hairpin RNA (shRNA) or tiplaxtinin treatment was associated with a marked inhibition of cellular proliferation causing a cell-cycle arrest in G1–S phase. The silencing of PAI-1 by either genetic or pharmacologic techniques conferred depletion of the G1–S transition molecules, cyclin D3/cdk4/6 and cyclin E/cdk2, with a concomitant increase in the cell-cycle inhibitors p21Cip1/Waf1 and p27Kip1. In addition, in vitro results were corroborated in a xenograft model...
in which silencing of PAI-1 led to a reduction in tumorigenicity with decreased levels of cyclin D3, and increased expression of p21\(^{\text{Cip1/Waf1}}\) and p27\(^{\text{kip1}}\), whereas overexpression of PAI-1 in HeLa xenografts led to an enhancement in tumor growth, which was associated with increased cyclin D3 expression and a decrease in expression of p21\(^{\text{Cip1/Waf1}}\) and p27\(^{\text{kip1}}\). Finally, immunohistochemical analysis of bladder and cervical tissues revealed that PAI-1 levels were increased in both human urothelial and cervical cancer patient tissue samples, and higher expression was correlated with more aggressive tumors.

**Materials and Methods**

**Immunohistochemical staining of human tumors for PAI-1**

After approval from the Institutional Review Board of MD Anderson Cancer Center Orlando (Orlando, FL), 4-μm tissue sections from 163 bladder cancer patient specimens and commercially available tissue microarrays (TMA) BNC12011 (US Biomax, Inc.), consisting of 37 benign bladder controls, as well as CR805 (US Biomax, Inc.), consisting of 70 cervical cancers and 10 benign cervical tissues, were examined by immunohistochemical staining. Details of the protocol and antibodies are available in Supplementary Material.

**Cell culture and reagents**

The human urothelial cell lines T24, UM-UC-14, RT4 (American Type Culture Collection, ATCC), and UROtsa, derived from benign urothelial tissue (a generous gift from Dr. Donald Sens, University of North Dakota School of Medicine, Grand Forks, ND) as well as the human cervical cancer cell line, HeLa (ATCC), were used for these studies and maintained in media as previously described (16, 17). T24, UM-UC-14, and HeLa cells were cultured in RPMI-1640 (ATCC) medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. RT4 cells were cultured in the McCoy medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen Corp.). Because of the difficulty in culturing RT4 cells, they were used in limited experimental assays. UROtsa cells were cultured in Dulbecco’s Modified Eagle Medium with low glucose (Invitrogen Corp.) supplemented with 1 mg/mL of glucose, 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. All cells were maintained in a standard humidified incubator at 37°C in 5% CO\(_2\), PAI-1 inhibitor, tiplaxtinin (PAI-039; Axon MedChem) was dissolved in dimethyl sulfoxide at a stock concentration of 10 mmol/L and stored at −20°C.

**Generation of PAI-1 knockdown/overexpression stable cell lines**

Stable cells containing a functional null knockdown of PAI-1 in T24 (T24-PAI-1\(^{\text{KD}}\)) and UM-UC-14 (UM-UC-14-PAI-1\(^{\text{KD}}\)) were generated using a plasmid with PAI-1 shRNA cloned within a pGFP-V-RS vector (OriGene Technologies) and transfected into T24 and UM-UC-14 cells at 80% confluency in a 6-well plate using Fugene HD transfection reagent (Roche Diagnostics). A plasmid with a scrambled (Scr) noneffective shRNA construct in pGFP-V-RS was concomitantly produced as a negative control (T24\(^{\text{Scr}}\) and UM-UC-14\(^{\text{Scr}}\)). The cells were selected in medium containing 1 mg/mL of puromycin (Life Technologies) for 14 days and subcloned by limiting dilution in 96-well plates. Pilot dose–response studies were performed to determine the lowest dose of G418 or puromycin that can kill all the untransfected cells. PAI-1 shRNA sequences are available in Supplementary Material. Next, HeLa cells stably overexpressing a functionally active PAI-1 (HeLa-PAI-1\(^{\text{OE}}\)) were produced using a plasmid containing a sequence verified human PAI-1 cDNA cloned into a pCMV6 entry vector and transfected into HeLa cells at 80% confluency in a 6-well plate using Fugene HD transfection reagent. A plasmid with vector alone was transfected as an empty control (HeLa\(^{\text{empty}}\)). The cells were selected in medium containing 1,200 mg/mL of G418 (Life Technologies) for 14 days and subcloned by limiting dilution in 96-well plates. All generated stable cell lines were maintained in media containing 0.25 μg/mL of puromycin for T24 clones (T24-PAI-1\(^{\text{KD}}\) and T24\(^{\text{Scr}}\)) and UM-UC-14 clones (UM-UC-14-PAI-1\(^{\text{KD}}\) and UM-UC-14\(^{\text{Scr}}\)) and in media containing 500 μg/mL of G418 for HeLa clones (HeLa-PAI-1\(^{\text{OE}}\) and HeLa\(^{\text{empty}}\)).

**Immunoblotting**

Cell lysate and immunoblotting were performed using standard protocols as previously described (16). Antibody details are available in Supplementary Material.

**Real-time reverse transcription PCR**

RNA was extracted from cells using the RNAeasy Mini Kit (Qiagen) as per the manufacturer’s instructions. Conversion to cDNA was achieved through the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative reverse transcriptase (RT) PCR was carried out using ABI 7300 Real-Time PCR System (Life Technologies) in a 20 μL reaction volume containing 1 μL of the first-strand cDNA, 1 μL of gene-specific TaqMan primer and probe mix. Primer sets can be found in Supplementary Material. The fold change in mRNA expression levels was calculated by the 2\(^{−ΔΔCt}\) method (18).

**Measurement of secreted PAI-1 by ELISA**

Parental tumor cells (T24, UM-UC-14, and HeLa) and PAI-1 clones (T24\(^{\text{scg}}\), T24-PAI-1\(^{\text{KD}}\), UM-UC-14\(^{\text{scg}}\), UM-UC-14-PAI-1\(^{\text{KD}}\), HeLa\(^{\text{empty}}\), and HeLa-PAI-1\(^{\text{OE}}\)) were plated onto 6-well plate at a density of 2 × 10\(^5\) cells in 2 mL of growth media per well. After incubation for 48 hours, the cultured supernatant was collected and centrifuged at 1,000 rpm for 5 minutes to remove any dead cells. The concentration of secreted PAI-1 in the culture supernatant was determined by using a plasminogen activator inhibitor 1 (PAI-1) Human ELISA Kit (ab99970; Abcam).
PAI-1 zymography

Of note, 30 μg of total cell lysate from PAI-1 clones (T24Scr, T24-PAI-1KD, UM-UC-14Scr, UM-UC-14-PAI-1KD, HeLaEmpty, and HeLa-PAI-1OE) and parental tumor cells (T24, UM-UC-14, and HeLa treated in the presence or absence of tiplaxtinin) were electrophoresed on 10% SDS–PAGE gel containing 1 mg/mL casein (Sigma), 10 μg/mL plasminogen (Sigma), and 0.5 mM/mL uPA under nonreducing conditions. After electrophoresis, SDS removal and PAI-1 renaturation were achieved by washing the gel for 1 hour in buffer containing 2.5% Triton-X 100, 50 mMol/L Tris pH 7.4, 5 mMol/L CaCl2, and 1 mMol/L ZnCl2. The gel was then incubated in a reaction buffer containing 50 mMol/L Tris pH 7.4, 5 mMol/L CaCl2, 1 mMol/L ZnCl2, and 0.02% Na3P3O10 pH 8.0 for 18 hours at 37°C, followed by staining with Coomassie blue. PAI-1 activity was indicated as zones of plasmin generation. Gel was photographed using the KODAK Gel Logic 200 Imaging System with Carestream Molecular Imaging Software Standard Edition v5.0.7.24 (Carestream Health) when opaque bands appeared on a clear background.

Proliferation and viability assay

PAI-1 clones (T24Scr, T24-PAI-1KD, UM-UC-14Scr, UM-UC-14-PAI-1KD, HeLaEmpty, and HeLa-PAI-1OE) and parental tumor cells (T24, UM-UC-14, and HeLa) were plated in white-walled 96-well dishes in triplicate (104 cells/well) and treated with or without tiplaxtinin at the indicated concentrations. Cell proliferation was determined by CellTiter-Glo Luminescent cell viability/proliferation assay (Promega) according to the manufacturer’s instructions at 24, 48, and 72 hours after seeding. Samples were incubated in an orbital shaker for 2 minutes to facilitate cell lysis followed by additional 10 minutes incubation at room temperature, and luminescence was measured using a FLUOstar Optima Reader (BMG LABTECH). IC50 of tiplaxtinin was determined in GraphPad Prism (GraphPad Software, Inc.) for each cell line. At least three independent experiments consisting of each condition tested in triplicate wells were used to calculate mean ± SD values.

Cell synchronization and cell-cycle distribution

For cell-cycle analysis, propidium iodide staining and fluorescence-activated cell sorting were performed in PAI-1 clones (T24Scr, T24-PAI-1KD, UM-UC-14Scr, UM-UC-14-PAI-1KD, HeLaEmpty, and HeLa-PAI-1OE) and parental tumor cell lines (T24, UM-UC-14, and HeLa), treated with or without tiplaxtinin. Briefly, cells were trypsinized, fixed in ethanol for 1 hour at 4°C, stained with propidium iodide solution (10 μg/mL) with RNase A (250 μg/mL), incubated at 37°C for 30 minutes, and analyzed using the BD FACScaliber with Cell Quest Pro Software (BD Biosciences) and ModFit LT (Verity Software House). Next to obtain populations of cells in G1-phase, the above cells were arrested by double thymidine block (19). Briefly, cells were blocked for 18 hours with 2.5 mMol/L thymidine, released for 8 hours by washing out the thymidine, and then arrested again with another 2.5 mMol/L thymidine for 18 hours. The cells were then released from the thymidine block with two washes of fresh medium and allowed to progress through G1-phase and into S-phase, then the cell-cycle position of the cells was determined at 0, 3, 6, and 12 hours by propidium iodide staining.

Subsequently, the progression into and through the S-phase was assessed in the above cells using the Click-IT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies). EdU (5-ethyl-2'-deoxyuridine) is a thymidine analog that becomes incorporated into DNA during active cellular DNA synthesis. Detection is determined via a copper-catalyzed covalent reaction between an azide (conjugated to Alexa Fluor 647) and an alkyne. Briefly, PAI-1 clones were seeded at 105 cells per well in a 6-well plate and subsequently treated with 10 μM of EdU for 24 hours before harvesting. Cells were trypsinized, fixed in 4% formaldehyde, and EdU incorporation was determined with the Click-IT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Molecular Probes) according to the manufacturer’s instructions. Cell-cycle distribution and DNA synthesis of synchronized cells were also assessed by flow cytometry using the BD FACSCaliber with Cell Quest Pro Software (BD Biosciences) and ModFit LT (Verity Software House) or FlowJo (TreeStar Inc.).

In vivo tumorigenicity

The importance of PAI-1 expression in regard to tumorigenicity was assessed in an in vivo mouse model. Athymic BALB/c (nu/nu) mice, 6- to 8-week-old, were obtained from Harlan Laboratories. Animal care was in compliance with the recommendations of The Guide for Care and Use of Laboratory Animals (National Research Council) and approved by the Institutional Animal Care and Use Committee. Tumor growth of the T24 PAI-1 knockdown clone, T24-PAI-1KD-19, was compared with the T24Scr control, the UM-UC-14-PAI-1 knockdown clone, UM-UC-14-PAI-1KD-4, was compared with the UM-UC-14Scr control, and finally the HeLa PAI-1 overexpression clone, HeLa-PAI-1OE-12, was compared with the HeLaEmpty control. Cells were harvested and resuspended in a 1:1 mixture of serum-free RPMI and Matrigel (BD Biosciences). T24 and UM-UC-14 cells (2 × 106) and HeLa cells (106) in 100 μL of RPMI-1640 were mixed with 100 μL of Matrigel and injected subcutaneously utilizing a 27-gauge needle into the rear flank of the mice as described previously (20). Tumor volumes were measured and recorded weekly with digital calipers and calculated by volume (mm3) = length × (width)2 × 0.5236. Statistical analysis of tumor volume was performed in GraphPad Prism (GraphPad Software, Inc.). After 4 weeks, mice were sacrificed and tumors resected for analysis.

Immunohistochemical analysis of xenograft tumors

Immunohistochemistry (IHC) was conducted as described in ref. 20. Details and antibodies listed in Supplementary Material.
Statistical analysis

Experimental data were expressed as mean with SD. All statistical analyses were conducted using the Student t test, the Mann–Whitney nonparametric U test, or the one-way ANOVA and compared with the control(s). The comparison between PAI-1 expression in human tumors, based on disease status, tumor grade, and tumor stage, was calculated using the Fisher exact test. A P value of <0.05 was considered significant. All statistical analyses and figures were carried out using GraphPad Prism software 5.0 (GraphPad Software, Inc.).

Results

PAI-1 expression is upregulated in aggressive human cancers

In biomarker discovery studies, our group previously profiled the transcriptome of exfoliated urothelia obtained from urine samples from a cohort of 92 patients with known bladder disease status. We identified PAI-1 as a valuable biomarker for the detection of bladder cancer (21). The PAI-1 expression correlation was validated in an independent cohort of 81 subjects using quantitative real-time PCR (22), and in two independent studies comprised of 435 subjects using ELISA assays (23, 24). In the present study, we assessed PAI-1 expression in human tissue specimens by immunostaining. Immunohistochemical staining of 37 benign bladder tissues and 163 bladder tumor tissues revealed significantly higher expression of PAI-1 predominantly within the epithelial component of bladder cancer tissues than in benign tissue (P < 0.0001; Fig. 1A–E). However, some immunoreactivity was noted in the stroma, mostly associated with vasculature. Furthermore, a correlation between PAI-1 expression status and clinicopathologic features of bladder cancer revealed a significant association of PAI-1 expression in muscle invasive bladder cancer (MIBC) compared with nonmuscle invasive bladder cancer (NMIBC; P = 0.0172; Fig. 1F). No association with tumor grade (P = 0.6021) was identified (Fig. 1G).

Next, we expanded our investigation to cancer of another organ, the cervix. A TMA consisting of 10 normal and 70 cervical cancers was analyzed for PAI-1 expression. PAI-1 was absent in benign cervical tissue, but significant (P < 0.0001) expression was observed in cancerous tissue (Supplementary Fig. S1). PAI-1 expression in cervical cancer specimens was located in the tumor cells predominantly, but was also associated with the vasculature in the tumor-adjacent stroma. Unlike in human bladder cancer, PAI-1 expression was not associated with higher stage cervical cancer (P = 0.3147); however, the expression was correlated with high-grade cervical cancer (P = 0.0303; Supplementary Fig. S1). The data provide evidence that increased PAI-1 levels are associated with aggressive cancers in bladder and cervical lesions.

Figure 1. PAI-1 overexpression in human bladder cancer. PAI-1 levels were measured by immunohistochemical analysis of 37 benign bladder and 163 bladder tumor tissues. Representative PAI-1 expression levels are shown in benign (A) and bladder cancer tissue samples with absent (B), weak (C), and strong (D) staining. Scale bars, 100 μm. Arrows depict stromal tissue. Correlation was assessed between PAI-1 expression among benign versus cancer tissue (E), NMIBC versus MIBC (F), and high-grade versus low-grade (G).
Downregulation of PAI-1 leads to inhibition of cellular proliferation

The baseline expression of PAI-1 was explored in a panel of cell lines: UROtsa, RT4, T24, UM-UC-14, and HeLa (Supplementary Fig. S2). Western blot analysis revealed that PAI-1 is differentially expressed in the panel, with increased expression apparent in T24 and UM-UC-14 cells. These results were confirmed at the mRNA level and by evaluation of secreted PAI-1 protein by ELISA. Given the progressive upregulation of PAI-1 in human bladder and cervical cancers, we investigated how PAI-1 influences key cell processes by genetically manipulating the expression of PAI-1 in T24, UM-UC-14, and HeLa cells. Briefly, we selected two stably transfected PAI-1 knockdown clones of urothelial T24 (T24-PAI-1KD-19 and T24-PAI-1KD-22) and UM-UC-14 cells (UM-UC-14-PAI-1KD-4 and UM-UC-14-PAI-1KD-17) using shRNA, and two stably transfected HeLa cervical cancer cells (HeLa-PAI-1OE-12 and HeLa-PAI-1OE-18) that overexpressed a functionally active human PAI-1. Efficiency of shRNA-mediated depletion and exogenous PAI-1 overexpression was documented by Western blot analysis, RT-PCR, and ELISA assay of the conditioned media (Supplementary Fig. S2). In addition to shRNA technology, inhibition of PAI-1 was achieved through treatment of parental T24, UM-UC-14, and HeLa cells with the small-molecule inhibitor, tiplaxtinin, a novel antagonist capable of silencing PAI-1 activity. PAI-1 activity was abrogated in urothelial parental cells and HeLa parental cells treated with tiplaxtinin, as demonstrated by the appearance of lytic zones of plasmogen generation in zymography gels containing plasminogen and uPA under nonreducing conditions. The expected activity of PAI-1 in urothelial cell knockdowns and overexpressing HeLa clones was confirmed by zymography (Supplementary Fig. S2).

In an in vitro proliferation assay, proliferation of T24 cells (Fig. 2A), UM-UC-14 cells (Fig. 2B), and HeLa cells (Fig. 2C) correlated with PAI-1 expression, i.e., reduced PAI-1 expression in T24 and UM-UC-14 clones was associated with a reduction in cellular proliferation. Treatment of all parental cells with tiplaxtinin resulted in a significant reduction in cellular proliferation (Fig. 2A–C). IC50 values for tiplaxtinin were determined for UROtsa cells (70.3 ± 0.1 μmol/L), T24 cells (43.7 ± 6.3 μmol/L), UM-UC-14 cells (52.8 ± 1.6 μmol/L), and HeLa cells (29.9 ± 3.1 μmol/L). Our results show that reduction of PAI-1 activity in T24, UM-UC-14, or HeLa cells via genetic manipulation or tiplaxtinin treatment causes significant cell growth inhibition.

Loss of PAI-1 causes cell-cycle arrest by differentially regulating key cell-cycle proteins

Next, we investigated the effects of silencing PAI-1 expression on cell-cycle progression. The percentage of cells in G0–G1, S, and G2–M phases of the cell cycle were monitored in cells with functional inhibition of PAI-1 (T24-PAI-1KD-19 and T24-PAI-1KD-22; UM-UC-14-PAI-1KD-4 and UM-UC-14-PAI-1KD-17), and in overexpressors of PAI-1 (HeLa-PAI-1OE-12 and HeLa-PAI-1OE-18) using flow cytometry (Fig. 3A). Inhibition of PAI-1 in T24 clones resulted in the accumulation of cells in G0–G1 phase from 65% in T24Scr control cells to 74% in T24-PAI-1KD-19 and 75% in T24-PAI-1KD-22, along with a reduction in S-phase cells from 29% in T24Scr control to 19% in both T24-PAI-1KD-19 and -22 clones. In UM-UC-14 clones, there was an accumulation of cells in G0–G1 phase from 55% in UM-UC-14Scr to 82% in UM-UC-14-PAI-1KD-4 and 73% in UM-UC-14-PAI-1KD-17, along with a reduction in S-phase cells from 28% in UM-UC-14Scr to 9% in UM-UC-14-PAI-1KD-4 and 3% in UMUC14-PAI-1KD-17.

Similar to PAI-1 knockdown, inhibition of PAI-1 with tiplaxtinin in parental cells caused a G0–G1 phase cell-cycle arrest (Fig. 3B). In T24 cells, tiplaxtinin treatment was associated with an increase in the percentage of cells in G0–G1 phase from 50% to 74% and a corresponding decrease in S-phase cells from 34% in T24 control cells to 8% in T24 cells treated with 50 μmol/L tiplaxtinin. UM-UC-14 cells exhibited an accumulation in G0–G1 phase from 59% in control cells to 76% in cells treated with 50 μmol/L tiplaxtinin, and depletion in cells in S-phase from 28% in control cells to 13% in tiplaxtinin-treated cells. In HeLa cells, G0–G1 phase, accumulation was observed from 57% in control cells to 75% in HeLa cells treated with 50 μmol/L tiplaxtinin, and a concomitant decrease in S-phase cells from 27% in control to 11% was observed.

To elucidate the molecular changes associated with the observed G0–G1 phase cell-cycle arrest in PAI-1–depleted cells, we assessed the expression of a panel of key regulatory proteins of the cell-cycle machinery by Western blot analysis (Fig. 3C and D). Inhibition of PAI-1 in T24-PAI-1KD or UM-UC-14KD clones and in parental T24, UM-UC-14, and HeLa cells treated with tiplaxtinin resulted in the depletion of the G1–S transition molecules cyclin D3, cdk4/6, cyclin E, and cdk2. A concomitant increase was observed in the levels of the cell-cycle inhibitors p53, phospho-p53, p21Cip1/Waf1, and p27Kip1, which all play significant roles in G0–G1 to S-phase progression. Thus, our data suggest that the loss of PAI-1 prevents cell-cycle progression of urothelial and cervical cancer cells through G1 into S-phase through the regulation of key cell-cycle factors.

PAI-1 knockdown interferes with progression through the G1-phase of the cell cycle

To further validate that abrogation of PAI-1 can lead to growth phase arrest of human cells, T24 clones (T24-PAI-1KD-19 and T24-PAI-1KD-22) and UM-UC-14 clones (UM-UC-14-PAI-1KD-4 and UMUC14-PAI-1KD-17) were synchronized in G0–G1 phase using a double thymidine block, then cell-cycle progression was monitored by flow cytometry following thymidine release. Although controls T24Scr and UM-UC-14Scr were able to progress out of G1-phase, T24 and UM-UC-14 clones depleted of PAI-1 were primarily arrested in G1-phase with little to no progression to S-phase (Fig. 4A). To confirm this finding, the percentage of cells entering S-phase was also monitored using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay.

Published OnlineFirst January 24, 2014; DOI: 10.1158/1541-7786.MCR-13-0543

Downloaded from mcr.aacrjournals.org on June 18, 2017. © 2014 American Association for Cancer Research.
Kit. Specifically, the incorporation of the thymidine analog, EdU was examined in cells at 0, 3, 6, and 12 hours after release from thymidine block (Fig. 4B). Depletion of PAI-1 did significantly delay the exit of cells from G1-phase, with only 11% and 15% of the T24-PAI-1KD-19 and T24-PAI-1KD-22 clone cells in S-phase, respectively, within 3 hours, whereas 43% of the T24Scr control cells progressed into S-phase ($P < 0.01$; Fig. 4B). In UM-UC-14 clones, only 10% of UM-UC-14-PAI-1KD-4 and 8% of UMUC14-PAI-1KD-17 cells were able to progress into S-phase, whereas 30% of UM-UC-14Scr control cells were in S-phase ($P < 0.01$; Fig. 4B). Our results suggest that PAI-1 expression is necessary to allow transition of cells from G1-phase to S-phase of the cell cycle. This is the first report that has linked PAI-1 to cell-cycle changes, and specifically assessed cell promotion through G0–G1 phase.

Inhibition of PAI-1 prevents xenograft tumor growth
Subcutaneous xenograft tumor growth of T24, UM-UC-14, and HeLa cells was assessed in athymic nude mice. In vivo experiments showed a marked decrease in tumor volume in the T24-PAI-1KD-19 and UM-UC-14-PAI-1KD-4 clones.
Figure 3. Inhibition of PAI-1 promotes cell-cycle arrest by depleting cell-cycle regulatory proteins associated with G1–S transition. A, cell-cycle progression was examined by flow cytometry in generated T24 clones (T24Scr, T24-PAI-1KD-19, and T24-PAI-1KD-22), UM-UC-14 clones (UM-UC-14Scr, UM-UC-14-PAI-1KD-4, and UM-UC-14-PAI-1KD-17) both depleted of PAI-1, in addition to HeLa clones (HeLaEmpty, HeLa-PAI-1OE-12, and HeLa-PAI-1OE-18) overexpressing PAI-1. B, furthermore, cell-cycle progression was noted in parental cells (T24, UM-UC-14, and HeLa) treated with tiplaxtinin. All experiments were repeated at least three times and a representative of one experiment is depicted. The percentage of cells in G0–G1, S, and G2–M phase of the cell cycle was observed by flow cytometry after propidium iodide staining of cellular DNA and analyzed by the ModFit software. Expression levels of several known G0–G1, S, and G2–M phase cell-cycle regulatory factors were analyzed by immunoblotting of clones in C and parental cells treated with tiplaxtinin in D.
knockdown clones when compared with their respective controls, T24Scr and UM-UC-14Scr (Fig. 5). Upon completion of the study at 4 weeks, T24Scr control xenografts were noted to have an average volume of 516 mm$^3$ compared with 168 mm$^3$ for T24-PAI-1KD-19 xenografts ($P = 0.0005$). UM-UC-14Scr xenografts had a volume of 1,550 mm$^3$ compared with 633 mm$^3$ for UM-UC-14-PAI-1KD-4 xenografts ($P = 0.0039$). Conversely, overexpression of PAI-1 in HeLa-PAI-1OE-12 enhanced tumor growth considerably relative to HeLaEmpty control cells. HeLaEmpty xenografts had a tumor volume of 933 mm$^3$ compared with HeLa-PAI-1OE-12, which exhibited a dramatic increase in tumor volume to 1,858 mm$^3$ ($P = 0.0195$; Fig. 5A). We confirmed the expected differences in tissue PAI-1 levels in...

Figure 4. Depletion of PAI-1 prevents passage of tumor cells out of G1 phase of cell cycle. A, T24 clones (T24Scr, T24-PAI-1KD-19, and T24-PAI-1KD-22) and UM-UC-14 clones (UM-UC-14Scr, UM-UC-14-PAI-1KD-4, and UMUC14-PAI-1KD-17) were synchronized in G0–G1 via a double thymidine block and cell-cycle progression was monitored following thymidine release by propidium iodide staining by flow cytometry analysis at 0 (orange), 3 (green), 6 (blue), and 12 hours (red). All experiments were repeated at least three times and a representative of one experiment is depicted. B, the percentage of cells entering S-phase was quantified using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit. Incorporation of EdU was examined in cells at 0, 3, 6, and 12 hours after release of thymidine block. All experiments were repeated at least three times. Data, mean $\pm$ SD. *$P < 0.05$, relative to control.
A

Tumor volume (mm$^3$)

Weeks after inoculation

T24$^{Scr}$

T24-PAI-1$^{KD}$-19

UM-UC-14$^{Scr}$

UM-UC-14-PAI-1$^{KD}$-4

HeLaEmpty

HeLa-PAI-1$^{OE}$

B

H&E

PAI-1

Cyclin D3

p27 Cip1/Waf1

p21kip1

UM-UC-14$^{Scr}$

UM-UC-14-PAI-1$^{KD}$-4

HeLaEmpty

HeLa-PAI-1$^{OE}$

C

Proliferation Index

T24$^{Scr}$

T24-PAI-1$^{KD}$-19

UM-UC-14$^{Scr}$

UM-UC-14-PAI-1$^{KD}$-4

HeLaEmpty

HeLa-PAI-1$^{OE}$

*
T24 and UM-UC-14 knockdown xenografts and HeLa overexpressor xenografts (Fig. 5B).

The expression levels of the proliferative marker (Ki-67) were also monitored in the excised xenograft tissues by IHC, and a proliferation index was quantified (Fig. 5C). We observed a significant reduction in proliferative capacity in T24-PAI-1 KD-19 and UM-UC-14-PAI-1 KD-4 tumors depleted of PAI-1, and an enhanced proliferative capacity in HeLa-PAI-1 OE-12. Specifically, T24-PAI-1 KD-19 showed a reduction in proliferative index of 91% compared with T24 Scr (P = 0.0007) and UM-UC-14-PAI-1 KD-4 exhibited a reduction of 72% compared with UM-UC-14 Scr (P < 0.0001). The overexpression of PAI-1 in HeLa-PAI-1 OE-12 led to a prominent increase in proliferation of 120% compared with HeLa Empty control (P = 0.0002; Fig. 5C). In addition, immunohistochemical analysis confirmed our in vitro cell-cycle data. Reduction of a functional PAI-1 in T24-PAI-1 KD-19 and UM-UC-14-PAI-1 KD-4 was accompanied by a dramatic reduction in the expression of cyclin D3 and an increase in the cell-cycle inhibitors, p21Cip1/Waf1 and p27Kip1 (Fig. 5D), alluding to a cell-cycle arrest in the G1-phase. Staining for HeLa-PAI-1 OE-12 xenografts is noted in Fig. 5B. Reduction of a functional PAI-1 in T24-PAI-1 KD-19 and UM-UC-14-PAI-1 KD-4 was not consistently accompanied by a reduction in the expression of cyclin E, cdk2 or p53 (data not shown).

Collectively, the xenograft tumor data show that the down-regulation of PAI-1 is associated with a diminution in tumor growth through the attenuation of proliferation coupled with the inhibition of key cyclin/CDK complexes and an induction of cell-cycle inhibitors involved in G1-S cell-cycle phase progression.

Discussion

PAI-1 is the primary inhibitor of tPA and uPA, and acts to suppress tissue and plasma fibrinolysis via plasmin conversion (25). PAI-1 is known to play a major role in benign disorders such as deep vein thrombosis, myocardial infarction, atherosclerosis, and stroke (26), and moreover, has been linked to some cancers (27, 28). Although PAI-1 activity has been associated with some tumor cell activities (7–9, 29–32), no data have linked PAI-1 to specific changes with cellular proliferation and the cell cycle to date.

Given our compelling data linking PAI-1 expression to cell proliferation, we focused our studies in further studying the role of PAI-1 in cell-cycle progression. In this study, we examined the tumorigenic and proliferative effects of PAI-1 on human urothelial and cervical cell lines by genetically altering the expression of PAI-1 and by interfering with PAI-1 activity with the small-molecule inhibitor tiplaxtin. In both in vitro and in vivo experiments, we found that the downregulation of PAI-1 led to (i) inhibition in cell proliferation and (ii) potent arrest in the G1-S phase of the cell cycle, a phenomena associated with reduction in cyclin D3/cdk4/6 and cyclin E/cdk2 and an increase in cell-cycle inhibitors, p53, p21Cip1/Waf1, and p27Kip1.

Cell proliferation relies on the activation of cyclins, which in turn bind to cyclin-dependent kinases (CDK), forming complexes that drive progression through the different phases of the cell cycle, and ultimately leads to cell growth (12, 13). This is coupled to regulatory mechanisms by CDK inhibitors that allow or disallow cells to progress through the cell cycle. Because uncontrolled cyclin/CDK activity is a well-established contributing factor for human cancer progression and tumorigenesis (13), we evaluated whether PAI-1 knockdown in tumor cells would affect cell-cycle development. Until now, there has been no evidence available showing the effects of functional PAI-1 as it relates to cell-cycle progression. Cyclin D members (D1, D2, and D3) are protooncogenic regulators of the G1–S phase checkpoint and have been linked with the pathogenesis of several tumor types (13). In addition, cyclin E and its binding partner cdk2 lead to the phosphorylation of downstream targets for growth progression (33–35). Our data suggest that PAI-1 may confer its protumor properties by causing cell progression through G1-phase via the upregulation of cyclin D3/cdk4/6 and cyclin E/cdk2 complexes.

In this study, the impairment of progression through G1 into S-phase in the PAI-1-depleted cells was also accompanied by prominent increases in the levels of the master cell-cycle regulator p53, and activation of specific downstream targets, including the CDK inhibitors, p21Cip1/Waf1 and p27Kip1, which both bind to and inhibit cyclin–CDK complexes to regulate cell-cycle progression through G1-phase. Following antimtotic signals, p21Cip1/Waf1 and p27Kip1 typically bind to cyclin–CDK complexes to inhibit their catalytic activity and induce cell-cycle arrest (36, 37). Cancer cells are known to tightly regulate such cell-cycle inhibitors to prevent arrest and thereby facilitate cell proliferation (38–40). Furthermore, the cyclin E/cdk2 complex is known to regulate p27Kip1 activity by flagging it for ubiquitination and subsequent degradation, which allows cyclin A expression and progression to S-phase (41). This perhaps explains the observed activation of p27Kip1 promoting G1–phase arrest of T24 and UM-UC-14 tumor cells in which PAI-1 was silenced.

TGF-β is known to arrest epithelial cells in G1-phase (42), whereas it is also known as a strong inducer of PAI-1 expression (43). Our data clearly illustrate that PAI-1 acts

Figure 5. PAI-1 knockdown results in reduction of xenograft tumor growth. A, tumor growth of human xenografts was assessed in vivo in athymic nude mice in the PAI-1 T24 cells (T24 Scr and T24-PAI-1 KD-19) and UM-UC-14 cells (UM-UC-14 Scr and UM-UC-14-PAI-1 KD-4). In addition, PAI-1 overexpressing cells (HeLa-PAI-1 OE-12 and HeLa Empty) were evaluated (n = 10/group). Tumor volumes were measured weekly for 4 weeks and plotted as mean ± SD. *, P < 0.05, relative to control. B, tumors were resected, fixed in 10% buffered formalin, and embedded in paraffin. Hematoxylin and eosin (H&E) images are included to identify and define tumor histology. Immunohistochemical analysis of xenograft tumors (T24 Scr, T24-PAI-1 KD-19, UM-UC-14 Scr, UM-UC-14-PAI-1 KD-4, HeLa Empty, and HeLa-PAI-1 OE-12) for PAI-1, cyclin D3, p21Cip1/Waf1, and p27Kip1 were conducted. Representative images from the four groups along are illustrated. These in vivo results confirmed our in vitro results; PAI-1 correlates with cyclin D3 expression and inversely correlates with p21Cip1/Waf1 and p27Kip1 expression. C, proliferative index was quantified on the basis of Ki-67 staining in each group. Data, mean ± SD. *, P < 0.05, relative to control.
as a G1-phase cell-cycle promoter and thus, generates some conflict with respect to the effect of TGF-β on cell-cycle progression. We are in the process of delving into this mechanism, but believe that PAI-1 and TGF-β interact with each other via a feedback loop (e.g., elevated TGF-β leads to elevated PAI-1, which then shuts down TGF-β). Furthermore, negative regulators in these pathways have been shown to be important, adding complexity and versatility to PAI-1 gene regulation (44).

Aberant expression of PAI-1 has been observed in some tumor types (e.g., breast and pancreatic) and has been associated with poor prognosis (45, 46). Little attention has been given to PAI-1 in human bladder or cervical tumors. For example, only two groups have reported on PAI-1 levels in patients with bladder cancer. Goodison and colleagues noted a significant increase in urothelial cell PAI-1 levels in patients bearing bladder tumors compared with non–tumor-bearing patients (22), and the same group reported increased urinary PAI-1 protein levels (23) in patients with bladder cancer in an independent study. Becker and colleagues reported significantly higher PAI-1 levels in tissue and plasma samples, but not in urine, from patients with bladder cancer compared with controls (47).

In cervical cancer, the literature on PAI-1 is even more scant. Kobayashi and colleagues demonstrated a significantly higher lymph node–positive rate in patients that had cervical tumors with strong urokinase plasminogen activator and/or PAI-1 tissue staining, than in those with tumors with weak expression (48). In another study, Tee and colleagues noted a specific PAI-1 allelic polymorphism in women with cervical cancer compared with women without cervical cancer (49). Thus, further exploration of PAI-1 in human tumors is warranted. Interestingly, PAI-1 expression was noted to be increased in MIIBC compared with NMIBC (Fig. 1F), but no association was noted with tumor grade (Fig. 1G). Furthermore, in cervical cancer, PAI-1 expression was not associated with higher stage cervical cancer, but was associated with high-grade cervical cancer (Supplementary Fig. S1). This stage- and grade-specific discrepancy could be due to inherited differences between bladder and cervical tumors, or if larger cohorts were studied, the discrepancies may be reduced or eliminated.

Recently, we reported that the reduction or inhibition of PAI-1 by tiplaxtinin resulted in the reduction of cellular proliferation, cell adhesion, and colony formation, and the induction of apoptosis and anoikis in vitro. Furthermore, treatment of T24 xenografts with tiplaxtinin resulted in inhibition of angiogenesis and induction of apoptosis, leading to a significant reduction in tumor growth. Similar results were obtained through evaluation of the human cervical cancer HeLa cell line, showing that PAI-1–mediated effects are not restricted to tumor cells of bladder origin (50).

References


Of note, in our in vitro studies when PAI-1 was silenced, the ability of cell to progress through the cell cycle was severely hampered (Fig. 4). However, we must point out that little expression of PAI-1 was evident in xenograft tumors in which PAI-1 was silenced. Despite genetically silencing PAI-1, xenograft tumors demonstrated a minimal growth potential over time, not a reduction in growth (Fig. 5). Thus, we believe that within xenograft tumors there are other factors that can help the tumors escape from the effects of silencing PAI-1, because these tumors still show some, albeit reduce proliferative potential. We are currently planning follow-up studies to closely look into this aspect of the project. Collectively, these data along with the present data show that targeting PAI-1 may be beneficial and support the notion that novel drugs such as tiplaxtinin could be investigated as anticancer agents. In this setting, care must be exercised when titrating tiplaxtinin to reduce any unsuspected bleeding issues.

Taken together, the results presented here show the importance of PAI-1 as it relates to tumor cell proliferation. We are the first to show that cell-cycle arrest associated with the downregulation of PAI-1 was accompanied by the depletion of G1 cell-cycle transition complexes in parallel with the upregulation of key cell-cycle inhibitors. Although its function is complex, further investigation into PAI-1 is warranted in the hope that it can provide insight into specific aspects of tumor biology and the identification of tumor cell vulnerabilities for therapeutic exploitation.

Disclosure of Potential Conflicts of Interest

S. Goodison is a CEO and has patent co-ownership in Nonagen Bioscience Corp. C.J. Rosser is a president and has ownership interest (including patents) in Nonagen Bioscience Corp—patents on bladder signature. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E.G. Giacoia, S. Goodison, C.J. Rosser
Development of methodology: E.G. Giacoia, M. Miyake
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.G. Giacoia, A. Lawton
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): E.G. Giacoia, A. Lawton, C.J. Rosser
Writing, review, and/or revision of the manuscript: E.G. Giacoia, S. Goodison, C.J. Rosser
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.G. Giacoia, M. Miyake, C.J. Rosser
Study supervision: S. Goodison, C.J. Rosser

Grant Support

This work is funded in part by the James and Esther King Biomedical Team Science Project, IKT-01 (to C.J. Rosser) and NIH/NCI RO1 CA108597 (to S. Goodison).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 11, 2013; revised December 4, 2013; accepted December 27, 2013; published OnlineFirst January 24, 2014.
PAI-1 Regulates Cyclin D3

www.aacrjournals.org
Mol Cancer Res; 12(3) March 2014 333

24. Wiman B, Collen D. Puri
tate tumor xenografts. Mol Cancer Ther 2007;6:101

25. Stefansson S, Haudenschild CC, Lawrence DA. Beyond fibrino-
lysis: the role of plasminogen activator inhibitor-1 and vitronectin

26. Fang H, Placencio VR, DeClerck YA. Protumorigenic activity of plas-
minogen activator inhibitor-1 through an apoptotic function. J Natl
Cancer Inst 2012;104:1470–84.

27. Wilkins-Port CE, Ye Q, Mazarukiewicz JE. Higgins PJ. TGF-beta1 +
EGF-initiated invasive potential in transformed human keratinocytes
is coupled to a plasmin/MMP-10/MMP-1-dependent collagen remodel-

al. Reduced metastasis of transgenic mammary cancer in urokinase-

29. Shapiro RL, Duquette JG, Roses DF, Nunez I, Harris MN, Camino H, et
al. Induction of primary cutaneous melanocytic neoplasms in
urokinase-type plasminogen activator (uPA)-deficient and wild-
type mice: cellular blue nevi invade but do not progress to malignant

30. Sabapathy KT, Pepper MS, Kiefer F, Möhle-Steinlein U, Tacchini-
formation: the role of the plasminogen activator/plasmin system. J Cell

31. Kraus M, Collen D, Gerard RD. Both u-PA inhibition and vitro-
nectin binding by plasminogen activator inhibitor 1 regulate

32. Stamatakos M, Palla V, Karaikos I, Xiromeritis K, Alexiou I, Pateras I,

33. Donnellan R, Chetty R. Cyclin E in human cancers. FASEB J

34. Malumbres M, Barbacid M. Cell cycle, CDKs, and cancer: a changing

35. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its

36. Gigliotto G, Mottl ML, Fusco A. Understanding p27(kip1) deregulation
in cancer: downregulation or mislocalization. Cell Cycle 2002;1:
394–400.

37. Nho RS, Sheaff RJ. p27kip1 contributions to cancer. Prog Cell Cycle

38. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function

39. Romanov VS, Pospelov VA, Pospelova TV. Cyclin-dependent kinase
inhibitor p21(Waf1): contemporary view on its role in senescence and

40. Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin

41. Duffy MJ. Urokinase plasminogen activator and its inhibitor, PAI-1, as
prognostic markers in breast cancer: from pilot to level 1 evidence

42. Ewen ME. p53-dependent repression of cycl4 expression in transform-
ing growth factor-beta-induced G1 cell-cycle arrest. J Lab Clin Med

43. Samarakoon R, Higgins PJ. Integration of non-SMAD and SMAD
signaling in TGF-beta1-induced plasminogen activator inhibitor type-1
gene expression in vascular smooth muscle cells. Thromb Haemost
2008;100:976–83.

44. Nagamine Y. Transcriptional regulation of the plasminogen activator
inhibitor type 1–with an emphasis on negative regulation. Thromb

45. Eljuga D, Razumovic JJ, Bulic K, Petrovecki M, Draca N, Bulic SO.
Prognostic importance of PAI-1 in node negative breast cancer
patients–results after 10 years of follow-up. Pathol Res Pract

expression of plasminogen activator inhibitor-2 (PAI-2) is a predictor of


### Molecular Cancer Research

**PAI-1 Leads to G₁-Phase Cell-Cycle Progression through Cyclin D3/CDK4/6 Upregulation**

Evan Gomes Giacoia, Makito Miyake, Adrienne Lawton, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-13-0543</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mcr.aacrjournals.org/content/suppl/2014/01/24/1541-7786.MCR-13-0543.DC1">http://mcr.aacrjournals.org/content/suppl/2014/01/24/1541-7786.MCR-13-0543.DC1</a></td>
</tr>
</tbody>
</table>

**Cited articles**

This article cites 50 articles, 16 of which you can access for free at: http://mcr.aacrjournals.org/content/12/3/322.full.html#ref-list-1

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.