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Inhibition of Apoptosis and Caspase-3 in Vascular Smooth Muscle Cells by Plasminogen Activator Inhibitor Type-1

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Abstract Increased expression of plasminogen activator inhibitor type 1 (PAI-1) is associated with decreased apoptosis of neoplastic cells. We sought to determine whether PAI-1 alters apoptosis in vascular smooth muscle cells (VSMC) and, if so, by what mechanisms. A twofold increase in the expression of PAI-1 was induced in VSMC from transgenic mice with the use of the SM-22α gene promoter (SM22-PAI$^+$). Cultured VSMC from SM22-PAI$^+$ mice were more resistant to apoptosis induced by tumor necrosis factor plus phorbol myristate acetate or palmitic acid compared with VSMC from negative control littermates. Both wild type (WT) and a stable active mutant form of PAI-1 (Active) inhibited caspase-3 amidolytic activity in cell lysates while a serpin-defective mutant (Mut) PAI-1 did not. Similarly, both WT and Active PAI-1 decreased amidolytic activity of purified caspase-3, whereas Mut PAI-1 did not. WT but not Mut PAI-1 decreased the cleavage of poly-[ADP-ribose]-polymerase (PARP), the physiological substrate of caspase-3. Noncovalent physical interaction between caspase-3 and PAI-1 was demonstrable with the use of both qualitative and quantitative in vitro binding assays. High affinity binding was eliminated by mutations that block PAI-1 serpin activity. Accordingly, attenuated apoptosis resulting from elevated expression of PAI-1 by VSMC may be attributable, at least in part, to reversible inhibition of caspase-3 by active PAI-1. J. Cell. Biochem. 92: 178–188, 2004.

Key words: plasminogen activator inhibitor type 1; caspase-3; apoptosis; serpin; vascular smooth muscle cells

The incidence of restenosis after percutaneous coronary intervention is increased markedly in patients with type 2 diabetes [Stein et al., 1995; Asakura et al., 1998; Elezi et al., 1998; Van Belle et al., 2001]. Both increased proliferation and decreased apoptosis of vascular smooth muscle cells (VSMC) contribute [Hanke et al., 1990; O'Brien et al., 1993; Bochaton-Piallat et al., 1995; Isner et al., 1995; Perlman et al., 1997; Bauriedel et al., 1998; Malik et al., 1998; Durand et al., 2002]. Expression of plasminogen activator inhibitor type 1 (PAI-1) is increased in the vessel walls of such patients and may contribute to the increased incidence of restenosis [Nordt et al., 1998; Sobel et al., 1998; Pandolfi et al., 2001]. Increased expression of PAI-1 is associated with resistance to apoptosis [Kwaan et al., 2000; Soeda et al., 2001; Gabriel et al., 2003]. This study was designed to determine whether increased expression of PAI-1 affects apoptosis in VSMC, and if so, to identify mechanisms responsible.

Increased caspase-3 activity induces apoptosis of VSMC [Wang and Keiser, 1998; Leitges et al., 2001; Orlandi et al., 2002]. However, caspases have pleiotropic effects. In lymphocytes, caspase activity mediates both apoptosis and proliferation [Kennedy et al., 1999; Boissonnas et al., 2002; Budd, 2002; Chun et al., 2002; Sturm et al., 2002]. In several types of cells, caspase-3 promotes differentiation [Zermati et al., 2001; Fernando et al., 2002; Kolbus et al., 2002; Sordet et al., 2002]. Recent observations suggest apoptosis and expression of PAI-1 may be linked through an interaction between caspase-3 and PAI-1. Activation of caspase-3 and induction of apoptosis of neurons were seen when cells were cultured in media deficient in PAI-1 [Soeda et al., 2001]. Further, addition of PAI-1 to culture media decreased apoptosis of neurons [Kwaan et al., 2000]. Thus,

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we postulated that PAI-1 might affect apoptosis of VSMC by directly interacting with caspase-3.

MATERIALS AND METHODS

Generation and Analysis of Transgenic Mice

The institutional animal care and use committee of the University of Vermont approved the use of animal and protocols. A chimeric gene construct in which the SM-22α promoter [Solway et al., 1995] was inserted upstream of murine PAI-1 cDNA (3.0 kb) was used to increase expression of PAI-1 by VSMC in transgenic mice. The presence of the transgene was determined in DNA isolated from tail biopsies with the use of slot–blot analysis and the polymerase chain reaction. All comparative analyses were performed with the use of transgene positive and negative littermates. Mice were fed a standard low fat (0.28% cholesterol) diet.

Expression of PAI-1 was compared in vessels fixed immediately after the mice (10–20 weeks of age) had been killed humanely and in VSMC that had been explanted from the same vessels. Immunohistochemical analysis of PAI-1 was performed as described previously [Taatjes et al., 2000]. Briefly, vessels were fixed with 3% formaldehyde in phosphate buffered saline (PBS) at 4°C for 16 h. Explanted VSMC were cultured on cover slips and fixed with 3% formaldehyde in PBS for 15 min. SYTOX® Green nucleic acid stain (Molecular Probes, Inc.) was used to identify nuclei, and a polyclonal rabbit anti-human PAI-1 antibody (a gracious gift from Prof. D. Collen) was used to detect PAI-1.

The concentration of PAI-1 in VSMC lysates was further determined with the use of an ELISA (MPAIKT-TOT™ kit, Innovative Research, Inc., Plymouth, MN).

Cell Cultures

VSMC were obtained by explantation from the aorta of littermates with (SM22-PAI$^+$) and without (control) the transgene as previously described [Schneider et al., 1997a]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Rockville, MD) supplemented with 20% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco-BRL), 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO), and insulin-transferrin-selenium (ITS, Gibco-BRL) at 37°C in atmosphere supplemented with 10% CO_2. Experiments were performed in DMEM with Hams' nutrient mixture F12 (DME/F12, Gibco-BRL). All experiments were performed with VSMC that had been maintained in culture for 2 to 8 passages.

The identity of smooth muscle cells was confirmed by Western blot analysis. Confluent cells were washed with cold PBS and lysed in 20 mM Tris-HCl, pH 7.4, 0.4 M KCl, 2 mM dithiothreitol, and 10% glycerol. Extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% mini-gel, transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad) and probed with a smooth muscle α-actin monoclonal antibody (1A4, Sigma) at a 1:1000 dilution for 2 h. Blots were washed and incubated with a secondary horseradish peroxidase-conjugated anti-mouse IgG (Sigma) for 1 h then processed for antibody visualization with the use of enhanced chemiluminescence detection reagents (Boehringer Mannheim) and imaged on Biomax film (Eastman Kodak). To confirm equal protein loading, blots were reprobed as above with a GAPDH monoclonal antibody (Research Diagnostics, Inc., Flanders, NJ).

Assessment of Apoptosis

VSMC (80% confluent) were exposed to DME/F12, or tumor necrosis factor (TNF, 10 ng/ml) plus phorbol myristate acetate (PMA, 20 ng/ml) [Chang and Tepperman, 2001; Siegmund et al., 2001] or palmitate (1 mM) [Dyntar et al., 2001; Listenberger et al., 2001] in DME/F12 for 24 h. Apoptosis was identified with the use of propidium iodide staining [Zamai et al., 2001] on whole cells and by determination of caspase-3 activity in lysed cell extracts.

Propidium iodide staining was performed with cells that were washed and resuspended in PBS. Subsequently, ice-cold ethanol was added to 80% v/v and cells were incubated at −20°C for 30 min. Fixed cells were briefly centrifuged then resuspended in a staining solution consisting of 10 μg/ml propidium iodide, 250 μg/ml RNase, and 1% fetal calf serum in PBS (Sigma). After an overnight incubation at 4°C in the dark, cells were analyzed by flow cytometry (Beckman Coulter, Epics XL, Miami, FL). The percentages of apoptotic cell nuclei (subdiploid DNA peak in the DNA fluorescence histogram) were calculated with System II software.

Caspase-3 activity was determined with the use of CaspaseTag™ caspase activity kits

(Intergen Co., Purchase, NY). Adherent cells were washed, detached, and resuspended in DME/F12. Cells were incubated with a carboxyfluorescein analog of benzyloxycarbonyl-aspartylglutamylvalylaspartic acid fluoromethyl ketone (FAM-DEVD-FMK) for 1 h at 37°C under 5% CO_2 in the dark. Cells were then washed and analyzed by flow cytometry.

Determination of Effects of PAI-1 on Caspase-3 Activity

To determine whether PAI-1 altered caspase-3 activity, cell lysates from VSMC were incubated with a physiologic concentration of PAI-1 in reaction buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT (HECD buffer, Sigma). Subsequently, the peptide substrate of caspase-3, acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (DEVD-pNA, Sigma), was added to the reaction and incubated at 37°C for 30 min. The release of *p*-nitroaniline was measured at 405 nm. Three types of PAI-1 were used, a wild type (WT) and two mutants [Gils et al., 1996; Gils et al., 1997; Nar et al., 2000] (kind gifts from Prof. P.J. Declerck). The inactive (Mut) PAI-1 mutant has point mutations in its active site (H190L–K191L) and does not inhibit plasminogen activators [Gils et al., 1997]. The active mutant (Active) has point mutations of N150H, K154T, Q301P, Q319L, and M354L. These mutations enable PAI-1 to maintain an active conformation rather than converting to an inactive latent conformation that occurs with WT PAI-1 [Nar et al., 2000]. To determine whether PAI-1 inhibits caspase-3 activity directly, purified recombinant human caspase-3 (Upstate Biotechnology, Lake Placid, NY) was incubated with PAI-1 proteins before determination of caspase-3 activity. Additional control experiments were performed with bovine serum albumin (BSA, Sigma), and the plasminogen activators, tissue type or urokinase type (tPA or uPA, American Dignostics).

The effect of PAI-1 on caspase-3 activity was characterized further by determining the effect of PAI-1 on caspase-3 induced cleavage of poly-[ADP-ribose]-polymerase (PARP), the physiological substrate of caspase-3 [Tewari et al., 1995]. The cleavage of PARP by caspase-3 was assessed with purified PARP (Alexis Biochemicals, Lausen, Switzerland) incubated in HECD buffer (Sigma) with or without PAI-1 protein for 20 min at 37°C. Subsequently, purified recombinant human caspase-3 was added to the reaction solution. Aliquots were removed at selected time-points, transferred into SDS–PAGE sample preparation buffer, and boiled for 3 min to quench the reaction. The proteins were separated by SDS–PAGE on a 6% mini-gel, transferred to PVDF membrane, and probed with a monoclonal antibody against PARP (Alexis Biochemicals) by Western blotting as described above. Intensity of bands was quantified with the use of Kodak software. Selected concentrations of PAI-1 (5:1, 10:1, 20:1, and 40:1 excess molar ratio to caspase-3) were used to delineate the effect of PAI-1 on caspase-3 activity. The concentrations of PAI-1, caspase-3, and their molar ratios were in the physiologic range reported in VSMC [Reilly and McFall, 1991; Robbie et al., 1996; Schneider et al., 1997b; Orlov et al., 1999].

Assessment of Physical Interactions Between PAI-1 and Caspase-3

To determine whether PAI-1 serves as a substrate for caspase-3 and whether PAI-1 forms a SDS-stable complex with caspase-3 (similar to that formed between PAI-1 and tissue type plasminogen activator or urokinase), purified recombinant caspase-3 protein was incubated with PAI-1 in HECD buffer. After a 20-min incubation at 37°C, proteins were separated by SDS–PAGE on a 10% mini-gel, transferred to PVDF membrane, and probed with a 1:5000 dilution of rabbit polyclonal antibody against PAI-1 by Western blotting.

Interaction between PAI-1 and caspase-3 was also assessed with antibody-immobolized PAI-1. A polyclonal goat anti-human PAI-1 antibody (American Diagnostics, Inc., Pendleton, IN) was exposed to Protein G-Sepharose (Pharmacia Biotech, Piscataway, NJ) and subsequently incubated with or without PAI-1 protein before addition of caspase-3 protein (1 μg for each reaction). The bead/protein mixture was washed four times in PBS plus 0.1% Tween-20. Bound proteins were resolved by SDS–PAGE and transferred to PVDF membrane. Caspase-3 was detected by immunoblotting with anti-caspase-3 polyclonal antibodies (Pharmingen, San Diego, CA).

A more quantitative method was used to further characterize the interaction between caspase-3 and PAI-1. Purified PAI-1 proteins (WT or Mut PAI-1) were diluted to 50 nM in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.5) with 5 μg/ml BSA and

applied to 96 well Costar® E1A plates (Corning) at 100 μl/well. Plates were incubated overnight at 4°C to allow passive adsorption of PAI-1 protein to the microtiter wells. The plates were washed twice with HBS containing 0.05% v/v Tween-20 and wells were blocked with HBS plus 2% w/v BSA for 1 h at room temperature. Under these conditions, WT and Mut PAI-1 proteins demonstrated equivalent coating efficiency when assayed by ELISA with the use of a rabbit polyclonal antibody to detect immobilized PAI-1 protein. To assess caspase-3 binding, selected amounts of purified His-tagged caspase-3 (100−0.1 nM) were diluted in binding buffer consisting of PBS plus 0.5% w/v BSA and applied to PAI-1-coated wells in triplicate at 100 μl/well. After a 1-h incubation, the plate was washed twice as above and 100 μl of a 1:10,000 dilution of HRP-conjugated Ni-NTA (Pierce Biotechnology) was added to each well to detect His-tagged caspase-3 bound to PAI-1. After a 1-h incubation at room temperature, the plate was washed twice, colorimetric substrate was added, and absorbance values at 490 nm were determined. BSA only-coated wells served as a negative control for caspase-3 binding.

Statistical Analysis

Results are means ± SD. Differences between two groups were identified with the use of Student's *t*-tests. For multiple groups, one-way analysis of variance and a Student–Newman-Keuls tests were used to identify differences. Significance was defined as $P < 0.05$.

RESULTS

Expression of PAI-1 in the Aorta and by VSMC From SM22-PAI$^+$ Mice

The expression of PAI-1 was increased in the aorta (fold induction = 3.0 ± 0.2, n = 6, $P < 0.001$, Fig. 1a) and explanted VSMC (fold = 2.1 ± 0.3, $P < 0.001$, Fig. 1b) from SM22-PAI$^+$ transgenic mice compared with negative control littermates as determined by semi-quantitative immunohistochemistry. Similarly, the concentration of PAI-1 in VSMC lysates determined with the use of an ELISA was 2.0 ± 0.3-fold greater in VSMC from SM22-PAI$^+$ transgenic mice compared with negative control littermates (Fig. 1c). Western blot detection of smooth muscle α-actin in VSMC lysates con-

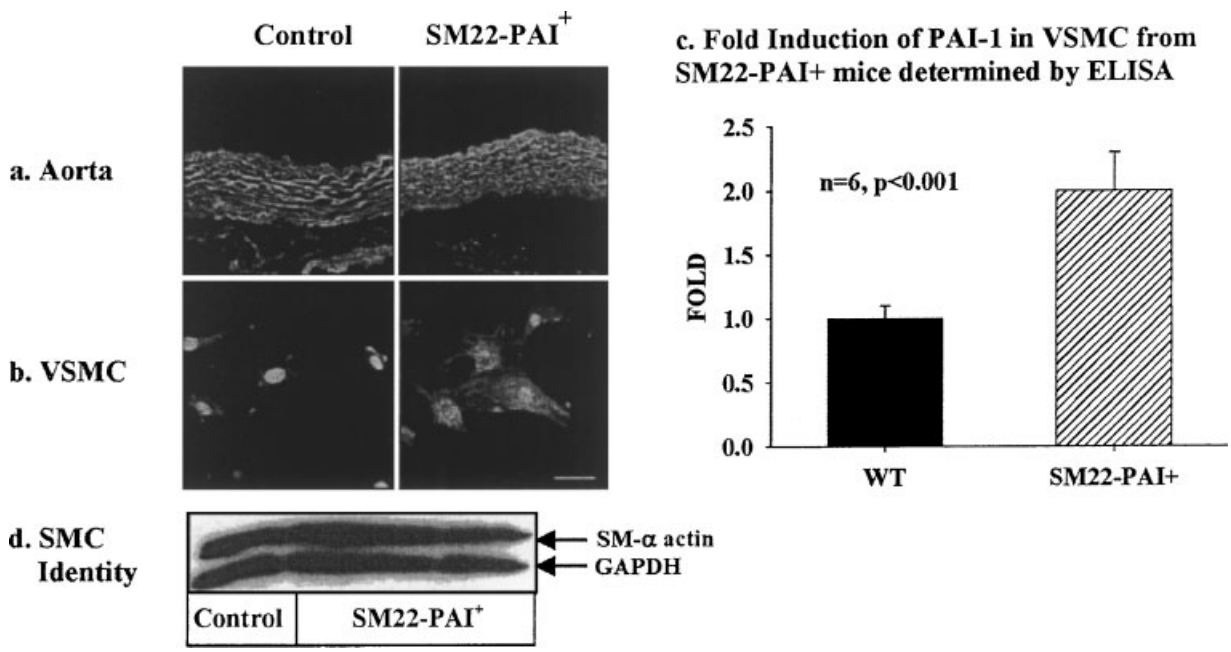


Fig. 1. Expression of PAI-1 in aortas (**a**, n = 6 pairs) and VSMC (**b**, n = 3 pairs) explanted from SM22 PAI$^+$ mice and control littermates. Immunostaining was performed with a rabbit anti-human PAI-1 antibody. SYTOX® green was used to identify nuclei. **c**: PAI-1 ELISA was used to confirm approximately twofold induction of PAI-1 by VSMC from SM22-PAI$^+$ mice compared with control littermates. **d**: Western blot shows comparable expression of smooth muscle α-actin in VSMC from control and SM22-PAI$^+$ mice. An anti-GAPDH antibody was used to verify equal loading.

firmed that explanted cells from SM22-PAI$^+$ mice maintained a differentiated phenotype in vitro (Fig. 1d). Moreover, flow cytometry revealed that >90% of cells explanted from either control or SM22-PAI$^+$ mice exhibited smooth muscle α-actin indicating that PAI-1 over-expression did not alter the state of VSMC differentiation.

Apoptosis of VSMC From SM22-PAI$^+$ Mice

When exposed to stimuli known to induce apoptosis, VSMC exhibited a typical phenotype of apoptosis that included cell shrinking, membrane blebbing, and development of chromatin condensation of nuclei. VSMC from control mice exhibited a greater prevalence of cell death after exposure to apoptotic stimuli. The percentage of cells in the subdiploid DNA peak after propidium iodide staining was greater in VSMC from control mice in response to 1 mM palmitate (control $= 14.9 \pm 4.5\%$ and SM22-PAI$^+ = 5.2 \pm 2.8\%$, n $= 6$, $P = 0.001$, Fig. 2a) and in response to 10 ng/ml tumor necrosis factor (TNF) plus 20 ng/ml phorbol myristate acetate (PMA) (TNF + PMA, control $= 46.6 \pm 10.9\%$ and SM22-PAI$^+ = 25.2 \pm 6.9\%$, n $= 4$, $P = 0.016$, Fig. 2a). The percentage of VSMC from SM22-PAI$^+$ mice that exhibited caspase-3 activity was less than that in VSMC from control mice when exposed to TNF + PMA (SM22-PAI$^+ = 23 \pm 5\%$ and control $= 41 \pm 3\%$, n $= 4$, $P = 0.001$, Fig. 2b) and palmitate (SM22-PAI$^+ = 10 \pm 4\%$ and control $= 24 \pm 2\%$, n $= 4$, $P < 0.001$, Fig. 2b).

Effect of PAI-1 on Caspase-3 Activity

To determine whether PAI-1 inhibits caspase-3 activity, recombinant wild type and two mutants of PAI-1 were added to cell lysates from VSMC and to purified recombinant caspase-3. Addition of WT PAI-1 and Active PAI-1 to cell lysates inhibited amidolytic activity of caspase-3 (by 45%, n $= 4$, $P = 0.002$ for WT PAI-1; by 50%, n $= 12$, $P < 0.001$ for Active PAI-1), but Mut PAI-1 had no effect (n $= 4$, Fig. 3a). Similarly, addition of WT and Active PAI-1 to purified recombinant caspase-3 decreased amidolytic activity of caspase-3 (by 22%, n $= 13$, $P < 0.001$ for WT PAI-1; by 33%, n $= 7$, $P < 0.001$ for Active PAI-1, Fig. 3b), whereas the Mut PAI-1 had no effect (n $= 7$). We found that caspase-3 activity was not affected by bovine serum albumin (BSA). Plasminogen activators, tissue type, or urokinase, did not cleave the peptide substrate of caspase-3. PAI-1 did not inhibit caspase-8 activity (data not shown). Therefore, the effect of PAI-1 on amidolytic activity of caspase-3 appears to be specific and independent of the effect of PAI-1 on plasminogen activators.

The inhibition of caspase-3 activity by PAI-1 was confirmed with the physiologic substrate of caspase-3, PARP. Addition of WT PAI-1 decreased the cleavage of PARP by caspase-3 (Fig. 4a). Mut PAI-1 had no effect at the same concentration (Fig. 4c). The inhibitory effect of

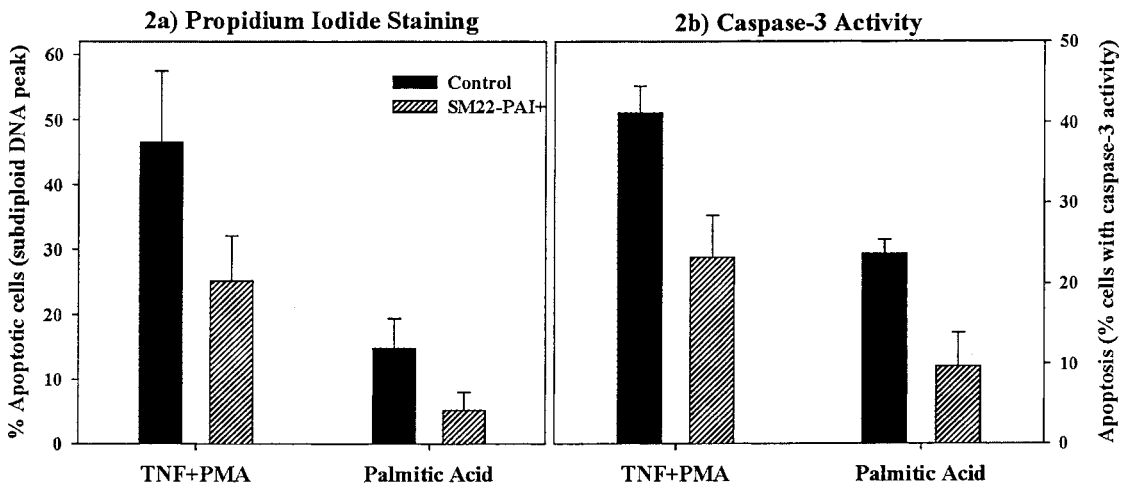


Fig. 2. Prevalence of apoptosis in VSMC explanted from control and SM22-PAI$^+$ mice after exposure to tumor necrosis factor (TNF, 10 ng/ml) plus phorbol myristate acetate (PMA, 20 ng/ml), or palmitate (1 mM). The prevalence of apoptosis was determined with the use of propidium iodide staining (**a**) or caspase-3 activity (**b**). a: The percentage of apoptotic cell nuclei (subdiploid DNA peak in the DNA fluorescence histogram) was calculated with the use of System II software. b: The percentage of cells with caspase-3 activity was identified with the fluorescent substrate FAM-DEVD-FMK. Bars show mean ± SD.

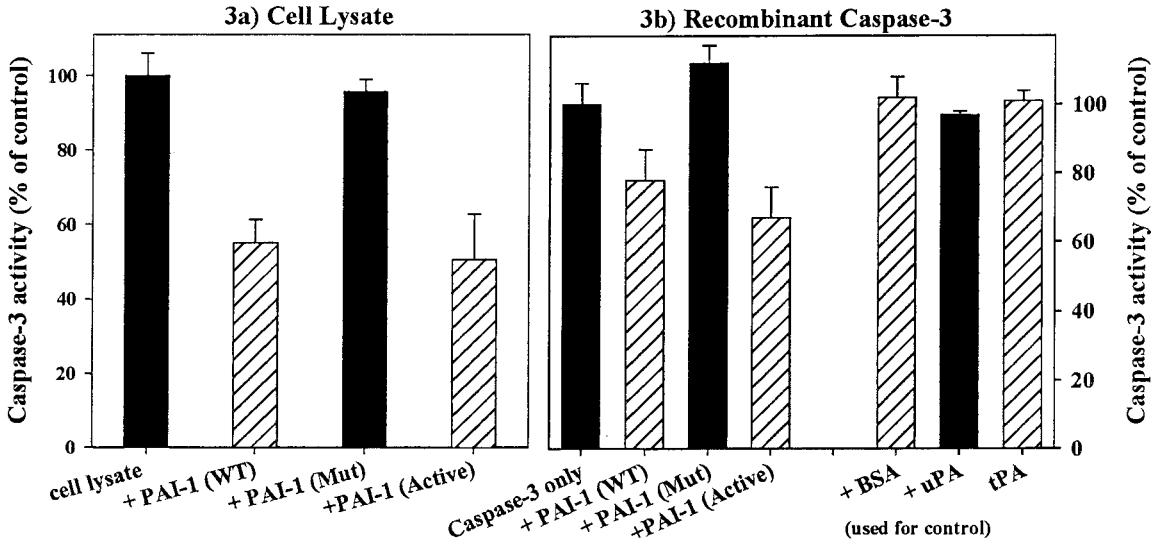


Fig. 3. Inhibition of caspase-3 activity by PAI-1. **a**: Protein extracted from VSMC (100 μg) was incubated with three forms of purified PAI-1 (800 nM): wide type (+PAI-1 WT), an inactive mutant (+PAI-1 Mut), and an active mutant PAI-1 (+PAI-1 active), or control conditions (cell lysate only), before addition of the peptide substrate of caspase-3, DEVD-pNA. Release of *p*-nitroaniline was measured at 405 nm. Results show caspase-3 activity as a percentage of control (mean ± SD). Both WT PAI-1 ($n = 4$, $P = 0.002$) and the stably active mutant ($n = 12$, $P < 0.001$) inhibited caspase-3 activity but the inactive mutant PAI-1 ($n = 4$) did not. **b**: Results from experiments in which recombinant human caspase-3 (20 nM) was incubated with PAI-1 (800 nM), control proteins (BSA, tPA, uPA) or control conditions (caspase-3 only) before addition of the peptide substrate of caspase-3, DEVD-pNA. Results show caspase-3 activity as a percentage of control (mean ± SD). Both WT ($n = 13$, $P < 0.001$) and stably active PAI-1 ($n = 7$, $P < 0.001$) inhibited caspase-3 activity, but not the inactive mutant PAI-1 ($n = 7$). BSA, tPA, and uPA had no effect on caspase-3 activity ($n = 4$ for each protein).

PAI-1 on caspase-3 was dependent on the concentration of PAI-1 (Fig. 4b).

Interaction Between PAI-1 and Caspase-3

PAI-1 forms a SDS stable complex with either uPA or tPA (Fig. 5a). To elucidate the nature of the interaction between PAI-1 and caspase-3, purified PAI-1 was incubated with caspase-3. PAI-1 did not serve as a substrate or form a SDS stable complex with caspase-3. No cleaved components of PAI-1 were evident on Western blots after incubation of PAI-1 with caspase-3 (Fig. 5a). No complex between PAI-1 and caspase-3 was identified with the use of Western blot analysis (performed under reducing and denaturing conditions, Fig. 5a). Interaction between PAI-1 and caspase-3 was characterized further with the use of antibody-immobilized PAI-1. A direct interaction between WT PAI-1 and caspase-3 was observed (Fig. 5b). Caspase-3 did not associate with Mut PAI-1 (Fig. 5b).

A comparative analysis of the interaction between caspase-3 and WT PAI-1 or Mut PAI-1 was conducted with the use of a more quantitative solid-phase binding assay to characterize binding affinity and specificity. Selected amounts of purified His-tagged caspase-3 were incubated with microtiter wells coated with either WT PAI-1 or Mut PAI-1. After removing free caspase-3, Ni-NTA-HRP was applied to detect bound caspase-3 with the use of a colorimetric assay (Fig. 5c). BSA only-coated wells served as a control to identify background binding of caspase-3. After subtraction of absorbance due to nonspecific binding, the corrected absorbance for WT and Mut PAI-1-coated wells were plotted with respect to the concentration of total caspase-3 applied. A concentration-dependent and saturable binding of caspase-3 to WT PAI-1 with an apparent Kd ~ 3 nM was demonstrated. Consistent with results of the enzyme activity assays, Mut PAI-1 demonstrated markedly reduced caspase-3 binding capacity. Accordingly, high affinity interaction between PAI-1 and caspase-3 requires an intact active site of PAI-1 that appears to facilitate reversible noncovalent association.

DISCUSSION

We have demonstrated that insertion of a chimeric gene SM22-PAI into mice increases expression of PAI-1 in the aorta and by VSMC explanted from transgene positive animals. The increased expression of PAI-1 by VSMC

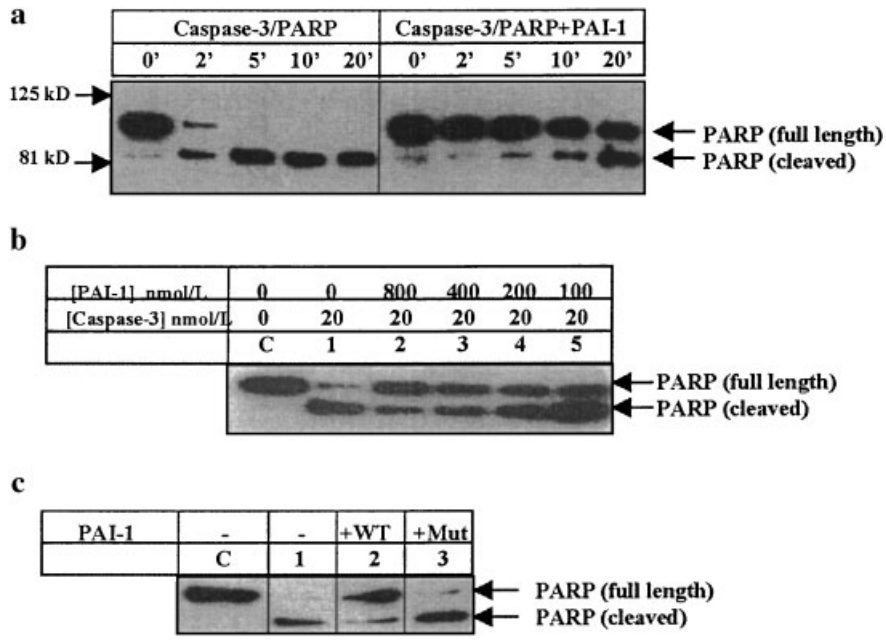
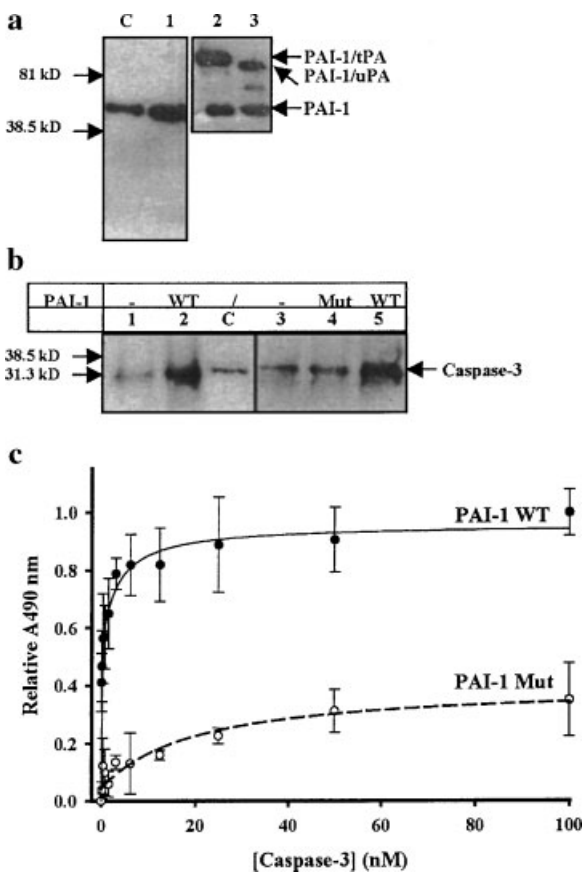


Fig. 4. Inhibition of caspase-3 mediated cleavage of PARP by PAI-1. **a**: Western blots demonstrate the cleavage pattern of PARP after addition of caspase-3 or caspase-3 plus WT PAI-1. PARP (100 ng) was incubated with or without PAI-1 protein (800 nM) for 20 min at 37°C. Subsequently, purified recombinant human caspase-3 (final concentration 20 nM) was added and samples were obtained at 2, 5, 10, and 20 min. Proteins were separated with 6% SDS–PAGE, transferred to PVDF membrane, and probed with a monoclonal antibody against PARP. Representative blots of four independent experiments are shown. Pretreatment with PAI-1 decreased the cleavage of PARP by caspase-3. **b**: The effect of selected concentrations of WT PAI-1 on PARP cleavage by caspase-3 was studied. Samples were obtained 10 min after addition of caspase-3. **Lane c** shows results with PARP protein alone. **Lane 1** shows results without addition of PAI-1. **Lanes 2–5** show results with a 40:1, 20:1, 10:1, and 5:1 molar excess of PAI-1 to caspase-3. Representative blots from three independent experiments are shown. PAI-1 inhibited the cleavage of PARP by caspase-3 in a concentration-dependent manner. **c**: The active site of PAI-1 is necessary for inhibition of caspase-3. PARP was incubated with or without equal concentrations of WT or Mut PAI-1 protein (200 nM) for 20 min at 37°C before addition of recombinant human caspase-3 (20 nM). Lane C shows results with PARP alone. Lane 1 shows results without addition of PAI-1 protein. Lanes 2 and 3 show results after addition of equal concentrations of WT (lane 2) or Mut (lane 3) PAI-1 to PARP. Representative blots from three independent experiments are shown. WT PAI-1 decreased the cleavage of PARP by caspase-3, whereas Mut PAI-1 did not.

decreased apoptosis of these cells. Further, we found that PAI-1 forms a high affinity complex with caspase-3 and thereby inhibits the functional activity of caspase-3. Accordingly, increased expression of PAI-1 limits apoptosis by a mechanism attributable, at least in part, to direct inhibition of caspase-3 by active PAI-1.

Our results demonstrating resistance to apoptosis of VSMC from SM22-PAI^{+} mice compared with that from control mice are consistent with observations by other groups [Kwaan et al., 2000; Soeda et al., 2001; Gabriel et al., 2003]. Addition of PAI-1 to cell culture media inhibits spontaneous and induced apoptosis of multiple cell lines, including the human prostate cancer cell line PC-3, a human promyelocytic leukemia cell line HL-60, as well as non-malignant cells such as human umbilical vein endothelial cells and the human breast epithelial cell line MCF-10A [Kwaan et al., 2000]. Similarly, inhibition of apoptosis of neurons has been associated with increased exposure to PAI-1. The effect of PAI-1 is apparent after addition of PAI-1 to culture media [Soeda et al., 2001] or after induction of expression of PAI-1 with the use of transforming growth factor-β [Gabriel et al., 2003]. Induction of apoptosis has been observed when PC-12 neurons were exposed to culture media deficient in PAI-1 [Soeda et al., 2001]. By contrast, a recent study demonstrated that inhibition of vitronectin-dependent cell adhesion by PAI-1 was correlated with induction of apoptosis by human umbilical vein endothelial cell as well as VSMC [Al-Fakhri et al., 2003]. Accordingly, PAI-1 may affect apoptosis in diverse ways. Our results demonstrate a direct interaction between PAI-1 and caspase-3 in VSMC. In addition, alteration of adhesion may initiate or disrupt signals that induce apoptosis.

A direct effect of PAI-1 on caspase-3 is consistent with observations in cells exposed to culture media deficient in PAI-1 [Soeda et al., 2001]. Our results demonstrate that caspase-3 activity decreases when expression of PAI-1 is increased. Direct inhibitory effects of PAI-1 on the activity of caspase-3 was seen when PAI-1 was added to extracts of VSMC or to purified caspase-3. Inhibition of caspase-3 activity was apparent when assessed with a peptide substrate of caspase-3, DEVD-pNA, and with the physiologic substrate of caspase-3, PARP. By contrast, an inactive mutant PAI-1 with limited capacity to bind to plasminogen activators did not inhibit caspase-3 activity. These results demonstrate that the active site of PAI-1 is necessary for inhibition of caspase-3. The essential role of the active site of PAI-1 is consistent with a previous report demonstrating that the inhibitory activity of PAI-1 is necessary to inhibit apoptosis in HL-60 cells [Kwaan et al., 2000]. The lack of effect of PAI-1 on caspase-8 indicates that the interaction between PAI-1 and caspase-3 is relatively specific for caspase-3.



The sequence and structure of PAI-1 were compared with that of other inhibitors of caspase-3 such as the baculovirus p35 protein, Bcl-2, and Bcl-x_L [Cohen, 1997]. No similarities were evident with respect to PAI-1 and these inhibitors. Caspases tend to cleave their substrates after an aspartic acid residue (Asp) in the P1 position [Thornberry et al., 1997]. The reactive center of PAI-1 is Arg^{346}-Met^{347} (Val-Ser-Ala-Arg-Met). This reactive center is contained within the exposed "strained loop region" at the carboxy terminus of the molecule and serves as a pseudosubstrate for the target serine protease. Because the P1 residue of PAI-1 is arginine (Arg), inhibition of caspases by PAI-1 would not be predicted.

Other members of the family of serine proteinase inhibitors (serpins) have been shown to limit apoptosis by inhibiting caspase activity. They differ, however, from PAI-1. Examples include the orthopoxvirus cytokine response modifier A (CrmA) [Ray et al., 1992] and human intracellular proteinase inhibitor-9 (PI-9)

Fig. 5. Analyses of physical interaction between PAI-1 and caspase-3. **a**: SDS–PAGE and Western blot analysis was conducted after purified recombinant caspase-3 (0.3 μg) was incubated with PAI-1 (1 μg) for 20 min. Membranes were probed with a rabbit polyclonal antibody against PAI-1. As a positive control, SDS stable interaction between PAI-1 and tPA or uPA (0.3 μg each) was assessed in parallel and results are shown in **lane 2** (tPA) and **lane 3** (uPA). **Lane c** shows PAI-1 without caspase-3. **Lane 1** shows PAI-1 incubated with caspase-3. Representative blots of three independent experiments are shown. The results demonstrate that caspase-3 does not cleave PAI-1 nor does it form an SDS stable complex with PAI-1. **b**: Interaction between PAI-1 and caspase-3 was assessed with antibody immobilized PAI-1. A goat anti-human PAI-1 polyclonal antibody was bound to Protein G-Sepharose. PAI-1 was then added followed by caspase-3 (1 μg each). After four washes, bound proteins were resolved by SDS–PAGE. Immunoblotting was performed with the use of anti-caspase-3 antibodies. Lanes 1 and 3 show results with caspase-3 added to the anti-PAI-1: Protein G-Sepharose. Lanes 2 and **5** show results with caspase-3 added to WT PAI-1: anti-PAI-1: Protein G-Sepharose. **Lane 4** shows results with caspase-3 added to Mut PAI-1: anti-PAI-1: Protein G-Sepharose. Lane C shows purified caspase-3 (0.1 μg). Representative blots from four independent experiments are shown. **c**: The relative binding affinity of caspase-3 for WT and Mut PAI-1 was evaluated with the use of a quantitative colorimetric binding assay. The absorbance at 490 nm for WT and Mut PAI-1-coated wells determined for each caspase-3 concentration were corrected for nonspecific binding of caspase-3 to BSA only-coated wells and then normalized with respect to 100 nM caspase value (defined as 1). Results were analyzed with the use of GraphPad Prism 4 software to estimate an apparent Kd for the interaction between caspase-3 and WT PAI-1. Results shown are from three independent experiments conducted in triplicate.

[Annand et al., 1999]. CrmA and PI-9 exhibit characteristics of the serpin superfamily such as their structure and mode of action. They are distinguished from other serpins by their ability to interact with cysteine proteinases. CrmA is an effective inhibitor of caspase-1, 4, 6, and 8, but inhibits caspase-2, 3, 7, and 10 to a limited extent [Cohen, 1997]. The reactive loop center of CrmA (Leu-Val-Ala-**Asp**-Cys) has the predicted Asp at the crucial P1 interaction site [Ray et al., 1992]. PI-9 has a similar reactive loop center, but a glutamic acid (Glu) rather than Asp residue in the P_1 position (Val-Val-Ala-**Glu**^{340}-Cys). PI-9 interacts with the cysteine proteases caspase-1, caspase-4, and caspase-8, but not with caspase-3 [Annand et al., 1999]. Accordingly, other members of the serpin family such as CrmA and PI-9 interact with caspases. However, unlike CrmA and PI-9, PAI-1 is an effective inhibitor of caspase-3.

Serpins such as PAI-1 bind to the active site of their cognate proteinases (plasminogen activators) to form an inhibitory complex with 1:1 stoichiometry. The PAI-1:PA complex is acid stable and resistant to denaturants. By contrast, the complex between caspase-3 and PAI-1 is dissociated by reducing and denaturing conditions used to perform SDS–PAGE. Accordingly, the interaction between PAI-1 and caspase-3 is similar to the interaction between CrmA and caspase-1 [Komiyama et al., 1994] as well as that between PI-9 and caspase-1 or -4 [Annand et al., 1999]. Inhibition of the active site of PI-9 decreases interaction between PI-9 and caspase-1 or -4 [Annand et al., 1999]. Similarly, we found the active site of PAI-1 is necessary for the interaction between PAI-1 and caspase-3.

In summary, our results suggest that increased expression of PAI-1 limits apoptosis of VSMC in vitro, at least in part, by decreasing caspase-3 activity. These findings provide mechanistic insight regarding a potential role played by PAI-1 in vascular remodeling in vivo. For example, studies conducted in rodent models of atherosclerosis and arterial injury have yielded mixed results in terms of the pathogenic versus protective affects of PAI-1 [Sjoland et al., 2000; DeYoung et al., 2001; Ploplis and Castellino, 2001; Peng et al., 2002; De Waard et al., 2003]. Although such differences may be related to the arterial target and type of injury used to induce neointimal hyperplasia, it is apparent from our studies in vitro that relatively subtle changes in the expression of intracellular PAI-1 can have dramatic effects on the propensity of VSMC to live or die in response to apoptotic stimuli. A similar phenomenon may occur in vivo resulting in altered sensitivity of resident VSMC to signals promoting neointimal expansion in vivo. In patients with diabetes who exhibit over-expression of PAI-1, apoptosis might therefore be limited thereby promoting increased accumulation of VSMC in response to injury to the vessel wall. One consequence of this might be the increased incidence of restenosis after percutaneous coronary interventions. Accordingly, therapy designed to diminish the expression or activity of PAI-1 may constitute a novel prophylactic approach.

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