Augmentation of Proliferation of Vascular Smooth Muscle Cells by Plasminogen Activator Inhibitor Type 1

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Objective—Proliferation of vascular smooth muscle cells (VSMCs) contributes to restenosis after coronary intervention. We have shown previously that increased expression of plasminogen activator inhibitor type 1 (PAI-1) limits VSMC apoptosis. Because apoptosis and proliferation appear to be linked, we sought to determine whether increased PAI-1 would affect VSMC proliferation.

Methods and Results—VSMCs were explanted from control and transgenic mice (SM22-PAI−/−) in which VSMC expression of PAI-1 was increased. Increased growth of SM22-PAI−/−-VSMCs (2.3±0.4-fold) reflected, at least partially, increased proliferation. Greater expression of FLICE-like inhibitory protein (FLIP; 2.7-fold) and its cleaved active form were seen in SM22-PAI−/−-VSMCs. The balance between caspase-8 and FLIP favored proliferation in SM22-PAI−/−-VSMCs. Increased expression of NF-κB and activation of extracellular signal-regulated kinase (ERK) were demonstrated in SM22-PAI−/−-VSMCs (fold=NF-κB=2.2±0.1, fold=phosphorylated-ERK=1.6±0.1). Results were confirmed when expression of PAI-1 was increased by transfection. Inhibition of NF-κB and ERK attenuated proliferation in SM22-PAI−/−-VSMCs. Increased expression of PAI-1 promoted proliferation when VSMCs were exposed to tumor necrosis factor (TNF).

Conclusions—Increased expression of PAI-1 is associated with greater activity of FLIP that promotes VSMC proliferation through NF-κB and ERK. Thus, when vascular wall expression of PAI-1 is increased, restenosis after coronary intervention is likely to be potentiated by greater proliferation of VSMC and resistance to apoptosis. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: proliferation ■ VSMC ■ plasminogen activator inhibitor type 1 ■ FLIP ■ restenosis

Despite technological advances, restenosis remains an important limitation of coronary intervention, particularly in patients with diabetes.12 Proliferation of vascular smooth muscle cells (VSMCs) plays a pivotal role in restenosis after vessel injury associated with coronary intervention.13 Consistent with this observation, inhibition of VSMC proliferation decreases neointimal cellularity after balloon injury.5

In contrast to the role of VSMCs in restenosis, migration of VSMCs into the neointima is a determinant of plaque vulnerability.6,7 We have shown that increased expression of PAI type 1 (PAI-1) limits migration of VSMCs, a phenomenon that may result in promoting generation of plaques more prone to rupture.8 The present study was designed to determine whether increased PAI-1 influences the proliferation of VSMCs.

Increased expression of PAI-1 has been associated with cellular proliferation and restenosis; however, the mechanism(s) responsible have not been elucidated. Increased expression of PAI-1 is associated with greater proliferation of neoplastic cells.9,10 Expression of PAI-1 is increased in the vessel wall in patients with diabetes, a group particularly prone to exhibit restenosis.11,12 In mice and other laboratory animals, increased arterial wall expression of PAI-1 has been found to promote increased neointimal cellularity after vascular injury.13–15 By contrast, the cellular response to exogenous injury was significantly decreased after arterial injury in PAI-1−/− mice.14,16

Results of recent studies have suggested that the balance between the activity of FLICE-like inhibitory protein (FLIP) and caspase-8 determines whether selected signals lead to apoptosis or proliferation. FLIP diverts Fas-mediated signals from death to proliferation in lymphocytes.17,18 In addition, increased expression of FLIP decreases apoptosis of pancreatic β cells and increases their proliferation.19 We have shown previously that increased expression of PAI-1 inhibits apoptosis of VSMCs by directly inhibiting caspase-3.20 Because inhibition of caspase activity was found to increase expression of FLIP,21,22 we hypothesized that inhibition of caspase-3 by PAI-1 would increase expression or activation of FLIP. FLIP has been demonstrated to lead to activation of nuclear factor κB (NF-κB) and extracellular signal-regulated kinase (ERK) that promote proliferation.18 NF-κB is a key regulator of genes involved in cell activation, survival, and
proliferation. Activation of NF-κB induces VSMC proliferation,23 and inhibition of NF-κB inhibits smooth muscle cell proliferation and promotes apoptosis.24-26 ERK signaling influences cellular processes such as proliferation, differentiation, and cell cycle progression.26 Inhibition of ERK decreases the growth and proliferation of smooth muscle cells.27 Accordingly, we sought to determine whether increased expression of PAI-1 affected the expression and activation of FLIP and its downstream mediators NF-κB and ERK to promote cell survival/proliferation.

Materials and Methods

Cell Culture

VSMCs were obtained by explantation from the aortas of SM22-PAI1 mice that exhibit a 3-fold increased expression of PAI-120 and negative control littermates, and grown in Dulbecco Modified Eagle Medium (DMEM; Gibco-BRL) supplemented with 20% fetal bovine serum as we described previously.20 The identity of smooth muscle cells was confirmed by Western blot and flow cytometry with smooth muscle cell specific α-actin antibody.20 Experiments were performed with cells in DMEM with Hams nutrient mixture F12 (DME/F12; Gibco-BRL). Tumor necrosis factor (TNF) was purchased from Sigma. All experiments were performed with VSMCs maintained in culture for 2 to 8 passages.

Growth of VSMC was determined by cell counts performed in triplicate daily for 6 days with the use of flow cytometry (Beckman Coulter, Epics XL). Each VSMC line was characterized twice.

Inhibitors of ERK and NF-κB

VSMCs at 80% confluence were exposed to an inhibitor of MAPK (PD98059, 10 μmol/L, Calbiochem)27 and an inhibitor of NF-κB: aminium pyrrolidithiocarbamate (APDC, 50 μmol/L, Calbiochem)28 or control media. The accumulation and the proliferation of cells were determined 24 hours after the cells were exposed to inhibitors or control conditions.

Adenovirus-Mediated PAI-1 Gene Transfection

VSMCs at 50% confluence were infected with adenovirus containing PAI-1(AdPAI-1) or control (AdRR5 without PAI-1) adenovirus (kindly provided by Dr P Carmeliet, University of Belgium)29 at 200 virus particles per cell in serum-free media. After 5 hours, serum-containing growth medium was added. Media containing the adenovirus were removed after 12 hours and replaced with DME/F12 media. The proliferation of AdPAI-1–infected VSMCs was determined after 72 hours and compared with that of VSMC infected with AdRR5.

Determination of Proliferation of VSMCs

DNA synthesis was assayed by the incorporation of 5-bromo-2-deoxyuridine (BrDU)10 (ABSOLUTE-ST proliferation kit; Phoenix Flow Systems). Cells that were 50% to 70% confluent were pulse labeled 2 hours with BrdU and fixed in ice cold 70% ethanol overnight at -20°C. Photolysis of DNA at sites of BrdU incorporation was induced with ultraviolet light. Subsequent labeling with deoxynucleotide triphosphate was catalyzed with terminal deoxynucleotidyl transferase. BrdU incorporation was identified with a fluorescein-labeled anti-BrdU antibody by flow cytometry.

Cell proliferation was determined also by the dye dilution method with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described.80 VSMCs (1 × 105) were washed with PBS, exposed to 2 μmol/L CFSE (Sigma) for 10 minutes, and then diluted with DMEM plus 10% FBS before being washed 3 times. After incubation for 1 hour, VSMC were divided equally into 3 groups. The first group was analyzed immediately with the use of flow cytometry to delineate fluorescence associated with the parent cells. The other 2 groups were incubated for 48 hours. After dissociation from the culture plate, cells were analyzed with the use of flow cytometry (Beckman Coulter) and MODFIT software.

Western Blot

VSMCs that had been exposed to serum-free DME/F12 media or DME/F12 with TNF (10 ng/mL) were lysed in lysis buffer containing 20 mmol/L Tris-HCl, pH 7.4, 0.4 mol/L KCl, 2 mmol/L dithiothreitol, and 10% glycerol. Concentrations of protein were determined with Bicinchoninic acid kit (Sigma). Extracted proteins were separated through a sodium dodecyl sulfate polyacrylamide gel, transferred to polyvinylidene difluoride membrane (Bio-Rad), and incubated with anti-FLIP, anti–Caspase-8 (Alexis Biotechnology Inc), anti–NF-κB (Santa Cruz Biotech Inc), and anti-ERK, anti-pERK, anti-MAPK, anti-p-MAPK, anti-Raf-1, and anti-p-Raf-1 (Cell Signaling Technology) or anti-GAPDH monoclonal antibody (Research Diagnostics Inc, to confirm equal loading). The primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies (Sigma) and enhanced chemiluminescence detection reagents (Roche Applied Science). The density of bands was analyzed with the use of densitometry and Kodak software (Eastman Kodak).

Statistical Analysis

Results are mean±SD. Differences between groups were identified with the use of Student t test. Significance was defined as P<0.05.

Results

Growth and Proliferation of VSMCs From SM22-PAI1 Mice

We have previously generated PAI-1 transgenic mice with increased expression of PAI-1 in VSMCs and demonstrated that increased expression of PAI-1 renders VSMCs more resistant to apoptosis.8,20 In the present studies, greater proliferation was demonstrated in VSMCs from PAI-1 transgenic mice (SM22-PAI1). After 6 days in culture, the average number of SM22-PAI1 VSMCs was 2.3±0.4-fold greater than control VSMCs (Figure 1A, n=6, P<0.001). The greater accumulation of cells reflected greater proliferation of VSMCs from SM22-PAI1 mice when assessed by BrdU incorporation (Figure 1B, control=17±2% and SM22-PAI1=31±6%, n=4, P=0.006) or with the use of cell tracking dye. SM22-PAI1 VSMCs exhibited a 45±8% greater (n=4, P<0.001) rate of proliferation (Figure 1C).

Expression and Activation of FLIP, Caspase-8, NF-κB, and ERK

Increased intracellular expression and activity of FLIP was seen in SM22-PAI1–VSMCs compared control VSMCs (Figure 2A and 2B). The expression of FLIP in SM22-PAI1–VSMCs was 2.7-fold greater than that in control cells (Figure 2B, n=4, P<0.001). In addition, the cleaved active form of FLIP (p43), known to promote proliferation, was the predominant species of FLIP seen in VSMC from SM22-PAI1 mice (Figure 2A). By contrast, the full-length form was the predominant species in cells from controls.

Caspase-8 can activate caspase-3 to induce apoptosis or cleave FLIP to generate p3 that induces proliferation. The protein expression of caspase-8 by VSMCs from SM22-PAI1 mice and their control littermates was similar (Figure 2C). Cleavage of procaspase-8 was apparent in protein extracts from SM22-PAI1 VSMCs and from VSMCs explanted from control littermates. However, the ratio of the cleaved form to procaspase-8 was greater in VSMCs from SM22-PAI1 mice (Figure 2C and 2D, n=4, P<0.001).
NF-κB mediates the proliferative effect of FLIP. Expression of NF-κB was greater in SM22-PAI⁺ VSMCs under control condition (Figure 2A and 2B, fold induction = 2.2 ± 0.1, n = 6, P < 0.001). Thus, increased protein expression of NF-κB in SM22-PAI⁺ VSMC may contribute to the increased proliferation of these cells associated with increased expression and activation of FLIP.

A second mechanism by which FLIP may induce proliferation is through ERK signaling. ERK signaling appears to be critical in diverting FLIP-mediated death receptor–induced apoptosis signals such as TNF from apoptosis to cell survival or proliferation. The intracellular protein expression of nonphosphorylated Raf-1, ERK, and p38MAPK was similar in VSMCs from SM22-PAI⁺ mice and control littermates. Addition of TNF to culture media did not affect the expression of the nonphosphorylated molecules (Figure 3A). By contrast, activation (phosphorylation) of Raf-1 and ERK was increased in VSMCs from SM22-PAI⁺ mice compared with that from control littermates (Figure 3A through 3C, n = 3, P < 0.05). Moreover, activation of ERK (p-ERK) and Raf-1 (p-Raf-1) was increased by TNF in SM22-PAI⁺ VSMCs but not control VSMCs (Figure 3A through 3C, n = 3, P < 0.05). Activation of p38MAPK was similar in VSMCs from SM22-PAI⁺ mice and control littermates, and it was not affected by TNF. These results demonstrate that activation of ERK and Raf-1 is increased in VSMCs with increased expression of PAI-1 and that increased PAI-1 by VSMCs diverts the death signal of TNF to that for survival/proliferation.

Adenoviral Transfection of PAI-1
Consistent with our observation with SM22-PAI⁺ VSMCs, VSMCs in which expression of PAI-1 was increased by
adenovirus transfection (AdPAI-1) exhibited increased growth and proliferation compared with VSMCs infected with control virus (Figure 4A and 4B, n=1005, P<0.05). The expression and cleavage of FLIP was increased in VSMC infected with AdPAI-1 compared with control virus (Figure 4C and 4D, n=1005, P<0.05). Further, increased NF-κB and p-ERK was identified in AdPAI-1–infected cells (Figure 4C and 4D, n=1005, P<0.05).

Figure 2. Expression of FLIP, caspase-8, and NF-κB in VSMCs from SM22-PAI+ and control mice. A and B, Western blot analysis of the expression of FLIP and NF-κB in VSMCs from SM22-PAI+ mice and negative control littermates. A representative blot from 4 (FLIP) or 6 (NF-κB) independent experiments is shown in A. Density of bands was analyzed with the use of densitometry. After results were normalized for expression of GAPDH, the relative density of FLIP (total and cleaved forms) and NF-κB (p65) in VSMCs from control was defined as 1. The mean±SD of the fold increases of relative density of FLIP and NF-κB in VSMCs from SM22-PAI+ mice is shown in B. C and D, Western blot analysis of the expression of Caspase-8 in VSMCs from SM22-PAI+ mice and negative control littermates. A representative blot from 4 independent experiments is shown in C. The density of the cleaved forms of caspase-8 was compared with that of the pro-caspase-8 in each cell line to determine the ratio of cleaved caspase-8 to pro-caspase-8. The mean±SD of this ratio in VSMCs from SM22-PAI+ mice and their control littermates is shown.

Figure 3. Expression of ERK, Raf-1, and p38MAPK in VSMCs from SM22-PAI+ and control mice. A, Western blot analyses were performed with proteins extracted from SM22-PAI+ and control VSMCs exposed to control media or TNF (10 ng/mL in DMEM/F12). Antibodies against Raf-1, p-Raf-1, ERK, p-ERK, p38MAPK, and p-p38MAPK were used. Representative blot of 3 independent experiments is shown. B and C, The density of bands was analyzed with the use of densitometry and normalized to the density of GAPDH. The relative density of pRaf-1 and pERK in control VSMCs exposed to DMEM/F12 was defined as 1, respectively. The relative density of the pRaf-1 and pERK in control VSMC exposed to TNFs or in SM22-PAI+ VSMCs exposed to DMEM/F12 and TNF was compared with that in control VSMCs exposed to DMEM/F12. The mean±SD of 3 independent experiments is shown.
Inhibition of NF-κB and ERK

Inhibition of NF-κB decreased proliferation of VSMCs from control and SM22-PAI²/H11001 mice (Figure 5, n=3, P<0.001). Inhibition of ERK did not affect proliferation of control-VSMCs (Figure 5, n=3, P=NS) but did inhibit proliferation of SM22-PAI¹-VSMCs (Figure 5, n=3, P<0.05). Accordingly, these results demonstrate that NF-κB is a key mediator of VSMC proliferation in vitro and are consistent with our observation that increased expression of PAI-1 increases proliferation through both ERK and NF-κB.

Discussion

In the present study we demonstrated that increased expression of PAI-1 by VSMCs increases their proliferation. Previously we have reported that increased expression of PAI-1 renders VSMCs resistant to apoptosis.²⁰ The resistance to apoptosis and the induction of increased proliferation appear to be linked. PAI-1 inhibits directly the activity of caspase-3 but not caspase-8.²⁰ This inhibition of caspase-3 parallels increased expression and cleavage of FLIP and downstream regulators of proliferation such as NF-κB and ERK.

Our observation that increased expression of PAI-1 increases proliferation of VSMCs is consistent with those made with cells from rats,¹³,¹⁵ mice,¹⁴ and humans.³¹ Increased expression of PAI-1 increases neointimal formation after balloon injury of carotid arteries in rats¹³ and mice.¹⁴ Conversely, decreased expression of PAI-1 is associated with attenuation of VSMC proliferation.³² By contrast, Carmeliet and colleagues found that the proliferation of VSMCs after electrical injury was similar in wild-type compared with PAI-1 knockout mice.²⁹ Notably, PAI-1 was not detected in uninjured arteries from the wild-type mice.²⁹ Thus, the similar response to arterial injury in wild-type and PAI-1 knockout mice may reflect the lack of a substantial difference in the expression of PAI-1 in VSMCs in the two groups. Their
observation of decreased neointimal formation after injury when PAI-1 expression was restored with adenoviral gene transfer is consistent with our previous observation that increased expression of PAI-1 inhibits VSMC contribution to neointimal formation presumably by inhibiting migration. Differing effects of PAI-1 on the in vitro proliferation of aortic endothelial cells and VSMCs are likely to be a reflection of differences in cell type, culture conditions, genotypes, and experimental design. Nevertheless, these results are consistent with the observation that increased intracellular expression of PAI-1 promotes proliferation of VSMCs.

Interaction between signals that initiate apoptosis and proliferation have been observed in studies of lymphocytes and VSMCs. FLIP renders many types of cells resistant to death receptor–mediated apoptosis. The expression of both PAI-1 and FLIP are increased in highly proliferative cells consistent with a link between these two proteins and cell proliferation. Because FLIP is an enzymatically inactive homologue of caspase-8, the relative expression of FLIP and caspase-8 determines whether cells undergo apoptosis or proliferation. In TNF-induced apoptosis, procaspase-8 binds to the death effector domain and undergoes autocatalytic activation that generates an active heterotetramer consisting of two large (p20) and two small subunits (p10). Caspase-8 initiates apoptosis through cleavage of downstream substrates such as procaspase-3. FLIP can limit apoptosis by binding to death receptors thereby blocking activation of caspase-8.

We found that the expression of caspase-8 was similar in VSMCs from SM22-PAI mice and negative control littermates. This observation is consistent with previous work suggesting that expression of caspase-8 is quite stable. Minimal or no variation in the expression of caspase-8 has been seen in response to diverse stimuli. Modest changes in expression of FLIP appear to determine whether a cell proliferates or dies in response to selected stimuli. Thus, our finding of increased expression of FLIP in VSMCs with increased expression of PAI-1 is consistent with a pivotal role of FLIP in cell proliferation.

Decreased apoptosis and increased proliferation have been seen in lymphocytes from human subjects who were homozygous for deficiency of caspase-8. Similar results have been obtained in studies of mice lacking caspase-8 in their T-cell lineage. Thus, the absence of caspase-8 is associated with increased proliferation in lymphocytes, an observation consistent with our findings in VSMCs with constitutively increased expression of PAI-1.

Caspase-8 cleaves FLIP at Asp-376 to generate N-terminal FLIP (p43) and C-terminal FLIP (p12). FLIP in turn permits the cleavage of procaspase-8. The cleaved fragment of FLIP (p43) binds preferentially compared with caspase-8 to the death effector domain. Further, cleaved (p43) FLIP binds TNF-receptor–associate factor 2 (TRAF2) more effectively than full-length FLIP. The binding of TNF to TRAF2 promotes activation of NF-κB. We observed increased expression of the cleaved form of FLIP (p43) associated with increased intracellular expression of PAI-1, a phenomenon expected to promote proliferation.

Increased proliferation of VSMCs from SM22-PAI mice appears to be mediated, at least in part, by increased expression of NF-κB. Activation of NF-κB induces proliferation of VSMCs. Inhibition of NF-κB decreases proliferation in a variety of cells including hepatocytes, epithelial cells and VSMCs. In addition, activation of ERK promotes smooth muscle cell growth and proliferation. Our results demonstrate that increased expression of PAI-1 is associated with increased activation of ERK and Raf-1, but not p38MAPK. Further, increased expression of PAI-1 appears to divert the death signal of TNF to a signal that promotes proliferation (phosphorylation of ERK and Raf-1). Accordingly, we hypothesized that increased expression of PAI-1 that inhibits apoptosis and promotes cell survival/proliferation in VSMCs through FLIP and downstream mediators NF-κB and ERK (Figure 6).

In summary, we have demonstrated that increased expression of PAI-1 increases proliferation of VSMCs as well as rendering them more resistant to apoptosis. We have shown previously that PAI-1 attenuates apoptosis by inhibiting caspase-3. Inhibition of caspase-3 appears to promote caspase-8–mediated cleavage and activation of FLIP that promotes proliferation through induction of NF-κB and activation of ERK signaling. These results are consistent with a direct effect of PAI-1 on VSMC proliferation likely to contribute to restenosis in patients with conditions such as diabetes mellitus that are associated with increased expression of PAI-1 in vessel walls.

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Disclosure(s)

None.

References

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