Fibrillar Collagen Regulation of Plasminogen Activator Inhibitor-1 Is Involved in Altered Smooth Muscle Cell Migration

Shinji Tanaka, Hidenori Koyama, Takuya Ichii, Atsushi Shioi, Masayuki Hosoi, Elaine W. Raines, Yoshiki Nishizawa

Objective—Vascular smooth muscle cells (SMCs) cultured on polymerized type I collagen fibrils are arrested in the G₁ phase of the cell cycle, and their phenotypic markers and pattern of expressed genes are markedly altered. In this study, we examined polymerized collagen regulation of plasminogen activator inhibitor (PAI)-1 and its involvement in SMC migration.

Methods and Results—We demonstrate that secretion and cell surface accumulation of PAI-1 are suppressed in SMCs cultured on polymerized collagen compared with SMCs cultured on monomer collagen. SMCs replated on vitronectin after culture on monomer collagen result in PAI-1 accumulation at focal adhesions and colocalization with α₃β₁ integrins. In contrast, polymerized collagen inhibits PAI-1 accumulation at focal adhesions when the SMCs are replated on vitronectin. Furthermore, for SMCs cultured on polymerized collagen, platelet-derived growth factor–stimulated migration on vitronectin is enhanced by PAI-1, with its function counteracted by urinary plasminogen activator. Finally, exogenous addition of PAI-1 appears to partly restore platelet-derived growth factor–stimulated α₃β₁-dependent SMC migration that is specifically suppressed by polymerized collagen.

Conclusions—Polymerized type I collagen fibrils dynamically regulate PAI-1, which may be involved in altered α₃β₁ integrin–dependent SMC migration. (Arterioscler Thromb Vasc Biol. 2002;22:1573-1578.)

Key Words: atherosclerosis • vascular remodeling • extracellular matrix • α₃β₁ integrin • platelet-derived growth factor

Migration and proliferation of vascular smooth muscle cells (SMCs) from the media into the intima contribute to lesion progression in atherosclerosis and restenosis after balloon angioplasty.¹ SMCs in the normal media are surrounded by extracellular matrix molecules, including collagen types I, III, and IV and laminin. SMC interaction with these matrix components can significantly influence their ability to respond to growth factors and/or chemotactants and can promote the modulation of SMCs from a contractile to a synthetic phenotype.²,³

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Generation of pericellular plasmin by urinary plasminogen activator (uPA) with subsequent direct or indirect proteolysis of the extracellular matrix is thought to contribute to matrix remodeling and cellular migration.⁴ The local tissue levels of active uPA and plasmin are regulated by plasminogen activator inhibitor (PAI) types 1 and 2 (PAI-1 and PAI-2, respectively). Active uPA avidly binds to specific glycosylphosphatidylinositol-anchored cell surface receptors (uPARs),⁵ also promoting focused pericellular proteolysis. Inhibition of active uPA by complex formation with PAI-1 or PAI-2 results in the removal of urokinase-PAI-uPAR complexes from the cell surface,⁶ which may be associated with a dynamic regulatory role of PAI-1 in uPAR-mediated cell adhesion and release.⁷ PAI-1, independent of its ability to inhibit plasminogen activators, binds to vitronectin or α₃β₁ integrins and directly modulates vitronectin receptor–mediated cell adhesion and migration.⁸–¹¹ Moreover, PAI-1 levels in SMCs are shown to be upregulated in arteries after balloon injury,¹²,¹³ and PAI-1 is known to be abundantly expressed in human atherosclerotic lesions.¹⁴,¹⁵ Thus, PAI-1 expression may be involved in phenotypic alteration of the SMCs in the progression of atherosclerosis.

We demonstrate that SMCs are arrested in the G₁ phase on polymerized type I collagen fibrils in vitro, whereas monomer collagen supports SMC proliferation. On polymerized collagen, cyclin E–cyclin-dependent kinase 2 activity is sup-
pressed through the upregulation of p27<sup>Kip1</sup>, a cyclin-dependent kinase inhibitor. In vivo, collagen expression is associated with the upregulation of p27<sup>Kip1</sup> expression and inhibition of cell replication in an animal model of lesion formation, suggesting a potential role of fibrillar collagen in the regulation of SMC phenotype in the progression of atherosclerosis. Moreover, we have recently shown that a culture of SMCs on polymerized collagen mimics many of the features of SMCs in normal media and modulates SMC gene expression. In the present study, we have examined the effect of polymerized collagen on PAI-1 regulation and cell migration in human SMCs. We demonstrate that polymerized collagen regulation of PAI-1 is involved in altered α<sub>3</sub>β<sub>1</sub> integrin–dependent SMC migration stimulated by platelet-derived growth factor (PDGF).

## Methods

### Materials

Immunocytochemically prepared human PAI-1 (No. 1090, 2850±264 uPA U/mg), a recombinant human metabolically stable mutant form of PAI-1 (No. 1094, 11 600±850 uPA U/mg), and recombinant inactive human PAI-1 (No. 1096, <500 uPA U/mg) were purchased from American Diagnostica Inc. The activity of each PAI-1 preparation was determined as described below (PAI-1 and uPA assay). Chemically purified PAI-1 (No. 1090) was 66.4±18.7% (mean±SD) as effective as recombinant stable PAI-1 (No. 1094) in stimulating SMC migration. Thus, we used the stable form of PAI-1 for most of the experiments to examine the role of active PAI-1. Anti–PAI-1 monoclonal antibody (Nos. 379 and 380, which recognize active and inactive PAI-1, respectively), uPA (No. 124), and blocking anti-uPAR antibody (No. 3936) were also purchased from American Diagnostica Inc. Type I collagen (Vitrogen 100) was obtained from Collagen Corp; human vitronectin, from Takara Biomedicals; human fibronectin, from GIBCO-BRL, Life Technologies Inc; and anti–human α<sub>3</sub>β<sub>1</sub> integrin (LM609), from Chemicon International Inc. Recombinant human osteopontin was kindly provided by Dr C.M. Giachelli (University of Washington, Seattle). Recombinant PDGF-BB was purchased from Genzyme. Human angiotensin II was purchased from Sigma Chemical Co.

### Cells and Cell Culture

Human SMCs (umbilical artery origin), obtained from Cell System Co, were cultured as described. SMCs were cultured on the surface of the indicated collagen preparations, polymerized collagen fibrils, and monomer collagen film, prepared as described.

### Chemotaxis/Migration Assay

Chemotaxis/migration assays were performed in a modified Boyden chamber, as described previously, with the use of matrix-coated filters (10 µg/mL human vitronectin, 20 µg/mL human osteopontin, 100 µg/mL bovine type I monomer collagen, or 10 µg/mL human fibronectin) and 10 ng/mL PDGF-BB or 100 nmol/L angiotensin II as a chemoattractant.

### Cell Adhesion Assay

SMCs were plated for 30 minutes under the same conditions used for the migration assay. Attached cells were fixed in 3.7% formaldehyde and stained with 0.5% toluidine blue/3.7% formaldehyde. Adherent cells were directly counted or solubilized in 2% sodium dodecyl sulfate for measurement of 650-nm absorption with a spectrophotometer.

### PAI-1 and uPA Assay

Release of PAI-1 and uPA into culture media was measured by a PAI-1 ELISA kit (TintElize PAI-1, Biopool) and a uPA ELISA kit (IMUBIND uPA ELISA kit, American Diagnostica Inc). The PAI-1 ELISA kit detects active and inactive forms of PAI-1, as indicated in the manufacturer’s instructions. PAI-1 activity was measured by titrating samples with increasing amounts of uPA into a fixed volume of SMC-conditioned media, as originally described. The excess of uPA activity was quantified by a uPA assay kit (Chemicon International). PAI-1 activity was calculated from the intersection of the asymptote of the titration curve with the x-axis and was expressed as units of uPA inhibited.

### Flow Cytometry, Immunocytochemistry, and Confocal Microscopy

Flow cytometric analysis, immunocytochemistry, and confocal microscopic analysis were performed as described previously. For flow cytometry, SMCs were suspended with collagenase digestion. In preliminary experiments, 30 minutes of collagenase treatment did not affect the surface level of PAI-1 or uPAR in our system. For cell surface PAI-1 determination, No. 380 anti–PAI-1 antibody, which equally recognizes active and inactive PAI-1, was used.

### Statistical Analysis

All the experiments were repeated at least twice. Statistical analysis was performed by the Student t test or ANOVA combined with a multiple comparison (Scheffé-type) test for comparing groups. These statistical analyses were carried out with the use of Stat View IV software (SAS Institute).

### Results

**Polymerized Collagen Fibrils Dynamically Regulate uPA, uPAR, and PAI-1 Expression in Human SMCs**

We have demonstrated that the culture of SMCs on polymerized collagen fibrils arrests cells in the G<sub>1</sub> phase of the cell cycle and alters the pattern of expressed genes. In Figure 1, we examined possible regulation of PAI-1 in human SMCs cultured on different forms of type I collagen. Compared with SMCs cultured on monomer collagen, SMCs cultured on polymerized collagen secreted less PAI-1 than the culture media (Figure 1a). PAI-1 activity in conditioned media at 24 hours on monomer collagen (0.26±0.04 U/mL) was significantly (P<0.05, Student t test) higher than that on polymerized collagen (0.18±0.03 U/mL). Surface accumulation of PAI-1 was also significantly suppressed by culture on polymerized collagen, as determined by flow cytometry (Figure 1b). In our SMC system, compared with PAI-1 secretion, uPA secretion was less abundant (Figure 1a). Moreover, flow cytometric analyses showed that only 5% of our SMCs were positive for uPAR expression (Figure 1a). Polymerized collagen increased the expression of uPAR, but only 20% to 25% of the SMCs were positive for the receptor (Figure 1b). Thus, PAI-1 dominates uPA expression in this SMC system, and its expression level is suppressed by polymerized collagen.

PAI-1 is known to interact with vitronectin and may modulate the function of α<sub>3</sub>β<sub>1</sub> integrin, a cell surface vitronectin receptor. Because levels of PAI-1 are dynamically altered in SMCs cultured on polymerized collagen, we examined its distribution in SMCs on vitronectin after the culture of SMCs on monomer or polymerized collagen for 24 hours. Analysis of PAI-1 distribution after culture on monomer collagen demonstrated the accumulation of PAI-1 close to the leading edge and in focal adhesion sites, together with diffuse staining at the bottom surface of the SMCs on...
vitronectin (Figure 1c). However, after 24 hours on polymerized collagen, SMCs failed to accumulate PAI-1 at focal adhesion sites, and PAI-1 was granularly distributed at the bottom of the cells.

PAI-1 Induces PDGF-Stimulated SMC Migration on Vitronectin

PAI-1 has been reported to affect cell migration in vitro in various experimental systems with different results. We examined the effects of PAI-1 on SMC migration after the culture of cells on polymerized collagen for 24 hours, a condition under which cells are arrested in the G1 phase and mimic many of the characteristics of medial SMCs in vivo. Vitronectin-coated filters were used for migration; thus, SMC motility was dependent on integrin (data not shown). Exogenous addition of PAI-1 protein to SMCs dose-dependently increased PDGF-induced chemotaxis (Figure 2). A similar effect of PAI-1 was also observed on filters coated with osteopontin, another integrin–dependent ligand (Figure 2). As previously described, PAI-1 (10 μg/mL) also accelerated cellular motility on vitronectin in U937 monocytic cells by 2.55 ± 1.2-fold (mean ± SD).

To understand the mechanism underlying PAI-1–stimulated SMC migration, we first examined the effect of PAI-1 on SMC adhesion to vitronectin. As shown in Figure 3a, PAI-1 did not affect the adhesion of SMCs to vitronectin. However, focal adhesion formation on vitronectin, determined by immunostaining of integrins, was suppressed by treatment with 10 μg/mL PAI-1 (Figure 3b). Thus, PAI-1 appears to inhibit the integrity of SMC adhesion to vitronectin.

We next examined whether uPA could counteract the action of PAI-1 on PDGF-stimulated SMC migration. After culture of the cells on polymerized collagen, the ability of PAI-1 (10 μg/mL, equal to 232 nmol/L) to stimulate SMC migration on vitronectin was suppressed by the simultaneous addition of uPA, and 300 nmol/L uPA almost completely prevented focal adhesion formation of SMCs on vitronectin (Figure 3c). However, after 24 hours on polymerized collagen, SMCs failed to accumulate PAI-1 at focal adhesion sites, and PAI-1 was granularly distributed at the bottom of the cells.
abrogated the effect of PAI-1 (see Figure I, which can be accessed online at http://www.ahajournals.org/). In accordance with the low expression level of uPAR in our experimental system, blocking the anti-uPAR antibody did not influence the effects of PAI-1 on PDGF-stimulated SMC migration.

Colocalization of PAI-1 With αvβ3 Integrin in SMCs Cultured on Vitronectin

Given the accumulation of PAI-1 at focal adhesions and its ability to enhance PDGF-stimulated SMC migration on vitronectin, we sought to determine whether PAI-1 associates with αvβ3 integrins on vitronectin. Double immunostaining with anti-αvβ3 integrin and PAI-1 antibody revealed that both molecules are colocalized at focal adhesions on vitronectin after culture on monomer collagen for 24 hours (Figure 4a). Moreover, when SMCs were plated on PAI-1 (50 μg/mL)-coated polycarbonate filter for an additional 30 minutes. Each column shows mean ± SD (n=3), *P<0.05 vs control IgG by Student t test.

To determine whether polymerized collagen might modulate αvβ3 integrin–dependent SMC migration. After 24 hours on polymerized collagen, PDGF-stimulated migration on vitronectin was suppressed by >50% compared with SMCs cultured on monomer collagen (Figure 5a). A similar inhibitory effect was also observed for osteopontin-coated filters, another ligand for αvβ3 integrin, but was not observed with monomer type I collagen or fibronectin as support matrices (Figure 5a), implying a suppressive effect of polymerized collagen specifically on αvβ3 integrin–dependent SMC migration. In cells that had been cultured on polymerized collagen, exogenous addition 30 minutes before the migration assay of active PAI-1, but not the latent and inactive form, which also lacks vitronectin binding activity, significantly restored PDGF-stimulated chemotaxis on vitronectin (Figure 5b). In contrast, PAI-1 did not affect SMC migration on vitronectin in cells cultured on monomer collagen. Thus, polymerized collagen fibrils specifically suppress αvβ3 integrin–dependent SMC migration, and altered regulation of PAI-1 may, at least partly, be involved in this process. The effect of PAI-1 may be specific to PDGF-stimulated signals, inasmuch as PAI-1 failed to recover polymerized collagen suppression of angiotensin II–induced SMC migration (Figure II, which can be accessed online at http://www.ahajournals.org/).

Discussion

Polymerized Type I Collagen Suppresses PAI-1 Expression in Human SMCs

We have demonstrated that the phenotype of SMCs is markedly modulated by polymerized type I collagen fibrils in vitro. This modulation includes cell cycle arrest with alteration in the levels of cell cycle molecules,16 changes in
phenotypic markers, and an altered pattern of expressed genes. Collagen expression is also associated with the upregulation of p27Kip1, the cell cycle inhibitor, and with the inhibition of cell replication in an animal model of lesion formation. Thus, fibrillar collagen appears to be involved in the regulation of the SMC phenotype in the progression of atherosclerosis. In the present study, we show that PAI-1 expression is dynamically suppressed by polymerized collagen compared with monomer collagen. PAI-1 is induced in arterial SMCs by balloon injury and is known to be abundantly expressed in atherosclerotic lesions.

PAI-1 Interacts With α3β1 Integrins and Enhances PDGF-Stimulated SMC Migration on Vitronectin

In addition to its strong protease inhibitory action against uPA, PAI-1 binds to vitronectin or α3β1 integrins and may play a role in vitronectin receptor–mediated cell adhesion and migration. PAI-1 inhibits cell migration through blocking the binding of α3β1 integrin to vitronectin in rabbit SMCs. In vivo, PAI-1–null mice exhibit excessive intimal thickening in blood vessels after transluminal mechanical injury. Acute treatment of PAI-1–null mice with exogenous PAI-1 completely inhibits SMC attachment to vitronectin, whereas completely inhibits SMC attachment to vitronectin, whereas blocking anti-uPAR antibody has no effect (data not shown). Thus, uPAR appears not to be a major vitronectin receptor in our SMCs. Taken together, α3β1 integrin appears to be a specific target of polymerized collagen that could be involved in altered α3β1 integrin–dependent SMC migration. In summary, polymerized type I collagen fibrils regulate PAI-1 expression, which may modulate α3β1 integrin function. Our data further suggest the possibility that within the normal media, inhibitory conditions for SMC migration on vitronectin induced by surrounding type I collagen fibrils may be dynamically modulated by local release of uPA and PAI-1 from infiltrating macrophages and inflammatory cells present at all stages of atherosclerotic lesion development.

Acknowledgments

This work is supported by grants for scientific research (No. 11838014 to H.K., No. 13671197 to H.K., and No. 11694307 to H.K., Y.N., A.S., and E.W.R.) from the Ministry of Education, Science, and Culture, Japan; a grant from ONO Medical Research Foundation (to H.K.); a grant from Osaka Medical Research Foundation for Incurable Diseases (to H.K.); and a grant from the National Institutes of Health (HL-18645 to E.W.R.). The authors thank M. Monden, Osaka City University, for excellent technical assistance, and Dr C.M. Giachelli, University of Washington, Seattle, for providing us with osteopontin.

References


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Arterioscler Thromb Vasc Biol. 2002;22:1573-1578; originally published online June 27, 2002; doi: 10.1161/01.ATV.0000028002.60919.4D

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Legend to figures

**Figure I.** PAI-1 induction of SMC migration on vitronectin is counteracted by uPA

SMC cultured on polymerized collagen were preincubated with indicated concentrations of reagents for 30 min, and analyzed for PDGF (10 ng/ml)-directed migratory activity on vitronectin for 4 hours in Boyden chamber apparatus (mean ± SD, n = 4). Anti-uPAR antibody was utilized at 20 µg/ml. *, p< 0.05 vs. without PAI-1; †, p< 0.05 vs. PAI-1 without uPA

**Figure II.** Polymerized collagen suppresses angiotensin II-stimulated SMC migration on vitronectin, and its effect is not restored by PAI-1.

Serum-deprived SMC were cultured on monomer (open bars) or polymerized (closed bars) collagen for 24 hours, and analyzed for angiotensin II (Ang II)-stimulated chemotactic activity (mean ± SD, n=4) on vitronectin in the presence or absence of 10 µg/ml PAI-1. The average basal migration of SMC from culture on monomer collagen is expressed as 100%. ‡, p<0.05 vs. monomer collagen.
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