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ORIGINAL ARTICLE

Plasminogen activator inhibitor-1 and vitronectin expression level and stoichiometry regulate vascular smooth muscle cell migration through physiological collagen matrices

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Summary. *Background:* Vascular smooth muscle cell (VSMC) migration is a critical process in arterial remodeling. Purified plasminogen activator inhibitor-1 (PAI-1) is reported to both promote and inhibit VSMC migration on two-dimensional (D) surfaces. *Objective:* To determine the effects of PAI-1 and vitronectin (VN) expressed by VSMC themselves on migration through physiological collagen matrices. *Methods:* We studied migration of wild-type (WT), PAI-1-deficient, VN-deficient, PAI-1/VN doubly-deficient (DKO) and PAI-1-transgenic (Tg) VSMC through three-D collagen gels. *Results:* WT VSMC migrated significantly slower than PAI-1- and VN-deficient VSMC, but significantly faster than DKO VSMC. Experiments with recombinant PAI-1 suggested that basal VSMC PAI-1 expression inhibits migration by binding VN, which is secreted by VSMC and binds collagen. However, PAI-1-over-expressing Tg VSMC migrated faster than WT VSMC. Reconstitution experiments with recombinant PAI-1 mutants suggested that the pro-migratory effect of PAI-1 over-expression required its anti-plasminogen activator (PA) and LDL receptor-related protein (LRP) binding functions, but not VN binding. While promoting VSMC migration in the absence of PAI-1, VN inhibited the pro-migratory effect of active PAI-1. *Conclusions:* In isolation, VN and PAI-1 are each pro-migratory. However, via formation of a high-affinity, non-motogenic complex, PAI-1 and VN each buffers the other's pro-migratory effect. The level of PAI-1 expression by VSMC and the concentration of VN in extracellular matrix are critical determinants of whether PAI-1 and VN promote or inhibit migration. These findings help to rectify previously conflicting reports and suggest that PAI-1/VN stoichiometry plays an important role in VSMC migration and vascular remodeling.

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Intimal hyperplasia is a central process in acquired vascular diseases, such as atherosclerosis and restenosis after balloon angioplasty. A key step in intimal hyperplasia is the migration of vascular smooth muscle cells (VSMC) from the media, through the extracellular matrix (ECM) composed of collagen, elastin and multiple other components, into the intima. The plasminogen activation (PA) system plays a major role in the regulation of cell migration and the development of intimal hyperplasia [1,2]. Plasminogen activator inhibitor-1 (PAI-1) is the primary physiological inhibitor of tissue- and urinary-type plasminogen activators (t-PA and u-PA, respectively) and a major regulator of fibrinolysis [3]. PAI-1 is present in plasma, platelets, endothelial cells, VSMC and the ECM. PAI-1 expression in the vascular wall is increased in several human vascular diseases characterized by neointima formation, suggesting that PAI-1 may regulate the development of intimal hyperplasia [4,5]. PAI-1 binds vitronectin (VN), an adhesive glycoprotein present in ECM that plays key roles in cell adhesion and migration [6,7]. Binding of PAI-1 inhibits VN's interactions with its receptors on VSMC, thereby inhibiting VSMC adhesion and migration [8,9]. However, PAI-1 has also been reported to promote VSMC migration by binding to low-density lipoprotein receptor-related protein (LRP), which is expressed on the surface of VSMC [10]. When bound to VN, PAI-1's LRP binding site remains in an encrypted state that does not bind LRP [11,12]. Hence, PAI-1 and VN regulate each other's functions and are poised to play key roles in VSMC migration and intimal hyperplasia.

While PAI-1 can either promote or inhibit VSMC migration *in vitro*, depending on experimental conditions, the net effect of PAI-1 on VSMC migration *in vivo* is unknown. However, it is difficult to study VSMC migration *in vivo*. Intimal hyperplasia cannot be used as a surrogate because it depends not only on VSMC migration, but also on VSMC proliferation and apoptosis and other processes independent of VSMC. Prior *in vitro* studies of the roles of PAI-1 and VN in VSMC migration have involved two-dimensional (2-D) culture systems in which cells are seeded on plastic surfaces coated with a purified matrix molecule and purified PAI-1 is added [8–10]. Two-D cell culture systems, such as the modified Boyden chamber (MBC), are useful, but do not adequately reproduce the complex, three-D ECM in which VSMC migrate *in vivo* [13,14], nor does addition of recombinant PAI-1 to cells coated on VN or other purified matrix components give adequate insight into the functional significance of PAI-1 and VN produced by VSMC themselves. Within the vascular wall, an environment not directly accessible to plasma, the pool of PAI-1 produced by VSMC is likely to be a major determinant of the overall effect of PAI-1 on VSMC migration. However, the impact of PAI-1 expression by VSMC on their migration under physiological conditions has not been studied. To address these issues, we examined the migration of VSMC with variable levels of PAI-1 expression through three-D collagen matrices. Given that VSMC express VN *in vivo* [15], as well as the key role of VN in determining PAI-1 function, we also studied the effects of VSMC VN expression and ECM VN concentration on PAI-1's migratory properties.

Materials and methods

Proteins

Recombinant human PAI-1 was expressed and purified as described previously [16]. The following mutants were used: (i) PAI-1-14-1b (PAI-1 N150H, K154T, Q319L and M354I), which inhibits u-PA and t-PA and binds VN with wild-type (WT) activities [17]; however, unlike WT PAI-1 (which has a half-life of 1–2 h under physiological conditions), PAI-1-14-1b is resistant to conversion to the inactive (i.e. latent) form (half-life > 140 h), and hence is useful in experiments examining PAI-1 function over longer periods of time, as in the migration assay described below. Throughout the present study PAI-1-14-1b is referred to as 'PAI-1-WT'; (ii) PAI-1-R (T333R, A335R), a reactive center mutant that binds VN normally, but has no detectable anti-proteolytic activity and cannot assume a latent conformation [16]; (iii) PAI-1-AK (PAI-1 N150H, K154T, Q319L, M354I, R101A and Q123K), an active, stable mutant with no detectable VN binding [16,18]; and (iv) PAI-1-R76E, I91L, an active, stable mutant that does not bind LRP [19]. Latent human PAI-1 and mouse multimeric VN were from Molecular Innovations. Platelet-derived growth factor (PDGF)-BB and rat tail collagen type 1 were from Millipore.

Animals

C57Bl/6J mice were from Jackson Labs. C57Bl/6J-congenic PAI-1-deficient ($Pai1^{-/-}$) mice were a gift from Peter Carmeliet, University of Leuven, Leuven, Belgium [20]. C57Bl/6J-congenic VN-deficient ($Vn^{-/-}$) mice and PAI-1-transgenic (Tg) mice that over-express PAI-1 under the control of the CMV promoter were from David Ginsburg, University of Michigan [21,22]. Mice received standard chow. All experiments were approved by the University of Missouri Office of Animal Resources.

Cell culture

VSMC were isolated from mouse aortas and grown in culture as described previously [23]. VSMC (passage 2–3) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and gentamycin/amphotericin B. After achieving 70–90% confluency, cells were serum starved (0.2% FBS) overnight. Cells were harvested by trypsin digestion, washed and resuspended in 0.2% FBS for use in the three-D cell migration assay. To determine if genetic deletion of PAI-1, VN or both affected the general migration properties of cultured VSMC, we performed control experiments in which WT, PAI-1-deficient, VN-deficient and PAI-1/VN doubly-deficient VSMC were seeded on collagen-coated porous membranes in a modified Boyden chamber and VSMC migration through pores into the lower chamber was stimulated with PDGF-BB [24]. At 6 h after seeding of VSMC on collagen there were no significant differences in migration between the four groups of cells (data not shown), suggesting that genetic deletion of PAI-1 and/or VN did not significantly alter the general migration properties of VSMC.

Three-D cell migration assay

Collagen gels were prepared as described previously [25], except that the final collagen concentration was 2.2 mg mL^{-1}. Polymerizing collagen mixture (30 μL) was pipetted into the upper chambers of 24-well Transwell inserts (Corning) whose bottoms consisted of porous (8 μm pore diameter) membranes. After gels polymerized, DMEM medium (200 μL) containing 0.2% fetal bovine serum (FBS) and murine VSMC (10^5) were added to the upper chamber. Inserts were placed into lower chamber wells filled with DMEM (600 μL) containing 2.5% FBS and PDGF-BB (20 ng mL^{-1}), which stimulate VSMC migration through the collagen gel into the lower chamber. After 72 h of standard cell culture conditions, inserts were removed, collagen gels were scraped away and membranes were stained with Diff-Quick (Siemens Healthcare Diagnostics, Newark, DE, USA). The lower-chamber side of the membrane, to which cells that migrate through the collagen gel and pores adhere, was visualized en-face with a microscope and cells were counted. In some experiments VN or recombinant PAI-1 was added to polymerizing collagen solutions and upper chamber culture media.

Western blotting and ELISA

VSMC were grown to confluency. Conditioned media (CM) was removed and cells were washed twice with phosphate-buffered saline and lysed by addition of sodium dodecyl sulphate (SDS) solution [26]. The lysate was centrifuged to remove insoluble material. Total protein concentration of the supernatant was measured with the BCA reagent. Samples (30 μg total protein) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting with rabbit antibodies raised against murine PAI-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and beta-actin (Cell Signaling Technology), as described previously [27]. PAI-1 concentration in CM was measured using an ELISA for murine PAI-1 (Innovative Research). Results were normalized to total protein concentration of CM.

Data analyzes

Duplicate or triplicate wells were performed for each set of experimental conditions in each experiment and mean results were calculated. A control group consisting of WT or untreated cells was included in each experiment. As indicated, results are reported as % control. All experiments were performed at least in triplicate, with results reported as mean ± standard error of mean. Experimental groups were compared using two-tailed Student's *t*-test or one-way analysis of variance.

Results

Isolated deficiency PAI-1 or VN increases VSMC migration through collagen, whereas combined deficiency of PAI-1 and VN inhibits migration

To study the functions of PAI-1 and VN expressed by VSMC during migration through collagen, we compared the migration of VSMC isolated from WT, $Pai1^{-/-}$ and $Vn^{-/-}$ mice. PAI-1-deficient VSMC migrated significantly faster than WT VSMC (Fig. 1), suggesting that under physiological conditions the dominant effect of basal PAI-1 expression by VSMC is anti-migratory. VN-deficient VSMC also migrated significantly faster than WT VSMC, although significantly slower than PAI-1-deficient VSMC (Fig. 1). To examine the impact of combined PAI-1 and VN deficiency on VSMC migration, we crossed $Pai1^{-/-}$ mice and $Vn^{-/-}$ mice to generate double-knockout mice, from which we established cultured lines of VSMC. While VSMC lacking only PAI-1 or VN each migrated faster than WT VSMC, VSMC lacking both PAI-1 and VN migrated significantly slower than WT VSMC (Fig. 1). These results suggested that a basal level of PAI-1 expression by VSMC inhibited migration only if VSMC VN expression is preserved. However, genetic deletion of VN converted the effect of VSMC PAI-1 expression to pro-migratory, as evidenced by comparing the migratory properties of VN-deficient VSMC (which express PAI-1) to those of VSMC lacking both PAI-1 and VN. These results also suggested that basal VN expression inhibited VSMC migration when PAI-1 expression was preserved, but promoted migration when PAI-1 was genetically deleted, as evidenced by comparing the migratory properties of PAI-1-deficient VSMC (which express VN) to those of VSMC lacking both PAI-1 and VN.

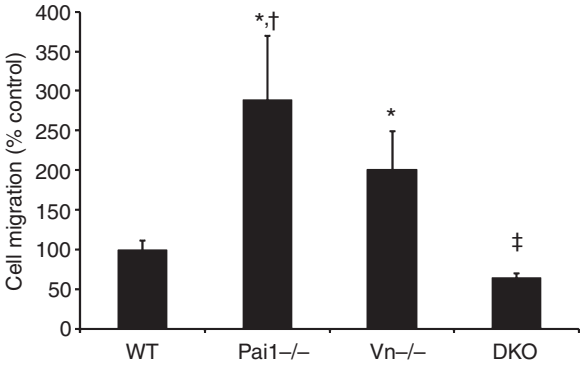

Fig. 1. Deficiency of plasminogen activator inhibitor-1 (PAI-1) or vitronectin (VN) enhances vascular smooth muscle cell (VSMC) migration, whereas combined PAI-1/VN deficiency inhibits migration. The number of VSMC migrating through the three-D collagen matrix in 72 h was determined, with results expressed as % control [i.e. wild-type (WT) VSMC]. $^{*}P < 0.001$ vs. WT; $^{\dagger}P < 0.01$ vs. $Vn^{-/-}$ VSMC; $^{\ddagger}P < 0.001$ vs. all other groups. DKO, double knockout genotype.

Recombinant PAI-1 mutants promote and inhibit VSMC migration through collagen by distinct mechanisms

We added several forms of recombinant PAI-1 (each at 1 μg mL^{-1}) to three-D migration assays containing PAI-1-deficient VSMC to enable us to study the impact of specific functional defects in PAI-1 on VSMC migration through collagen. PAI-1-WT significantly increased migration of PAI-1-deficient VSMC (Fig. 2). PAI-1-AK (which inhibits PAs, but does not bind VN) also significantly increased migration of PAI-1-deficient VSMC. However, PAI-1-R76E, I91L (which inhibits PAs and binds VN, but does not bind LRP) did not increase PAI-1-deficient VSMC migration, rather producing a significant inhibitory effect (Fig. 2). These results suggested

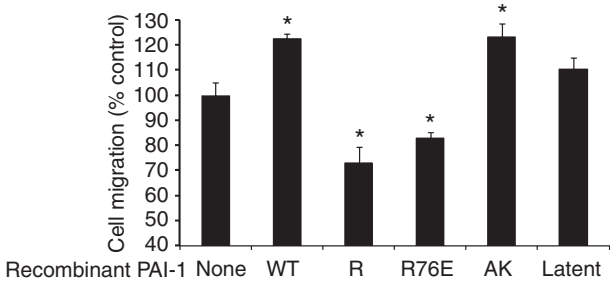

Fig. 2. Effects of recombinant plasminogen activator inhibitor-1 (PAI-1) on vascular smooth muscle cell (VSMC) migration. All cells used in this experiment were PAI-1 deficient. $^{*}P < 0.002$ vs. control PAI-1-deficient VSMC that were not treated with recombinant PAI-1 ('None' group). 'R76E' designates PAI-1-R76E, I91L.

that active PAI-1 can stimulate VSMC migration through collagen by a mechanism that requires LRP binding, but does not require binding to VN, which is secreted by VSMC and binds collagen [28]. PAI-1-R (which does not inhibit PAs, but binds VN with normal affinity) significantly inhibited migration of PAI-1-deficient VSMC (Fig. 2). Latent PAI-1, which does not inhibit PAs and has low binding affinity for VN, had no significant effect on migration of PAI-1-deficient VSMC. Together, these results suggested that PAI-1 can inhibit VSMC migration through collagen under physiological conditions by a VN-dependent mechanism that does not require protease inhibition or LRP binding.

We also studied the effect of selected forms of recombinant PAI-1 on migration of VN-deficient VSMC. PAI-1-WT significantly increased migration of VN-deficient VSMC (i.e. 1.4 ± 0.1-fold compared to control matrices lacking PAI-1-WT, $P < 0.001$), further suggesting that enhancement of VSMC migration by PAI-1-WT does not require VN binding. PAI-1-R had no significant effect on migration of VN-deficient VSMC (data not shown), further suggesting that inhibition of VSMC migration by PAI-1-R requires VN binding.

ECM PAI-1 and VN increase migration of WT VSMC, but each factor inhibits the other's pro-migratory effect

Concentrations of PAI-1 and VN in the vascular wall are increased in some human disease states [4,29]. We supplemented collagen matrices with PAI-1-WT (1 μg mL^{-1} = 23.3 nM), multimeric VN (10 μg mL^{-1} = 133 nM), or both PAI-1-WT and VN to examine the effects of increased ECM concentrations of these factors on VSMC migration. PAI-1-WT significantly increased migration of WT VSMC through collagen (Fig. 3, bar 1 vs. 2). VN also significantly increased migration of WT VSMC through collagen (Fig. 3, bar 1 vs. 3). These results suggested that increased an ECM concentration of either active PAI-1 or VN promotes VSMC migration. However, addition of both PAI-1-WT and VN to collagen matrices resulted in no significant change in VSMC migration compared with collagen matrices to which neither factor was added (Fig. 3, bar 1 vs. 4), suggesting that active PAI-1 and VN can each inhibit the pro-migratory effect of the other. Consistent with this hypothesis, addition of VN (10 μg mL^{-1}) to collagen matrices reduced the migration of VN-deficient VSMC (which express PAI-1) by 57.2 ± 6.6% ($n = 3$, $P < 0.001$). We also examined the effect of adding a higher concentration of PAI-1-WT (5 μg mL^{-1} = 116 nM) along with VN (10 μg mL^{-1}) on migration of WT VSMC. Under these conditions VSMC migration was significantly increased compared with control conditions lacking addition of either factor (Fig. 3, bar 1 vs. 5). These results suggested that a stimulatory effect of active PAI-1 on VSMC migration can be observed in the presence of VN if the PAI-1 concentration is sufficiently high.

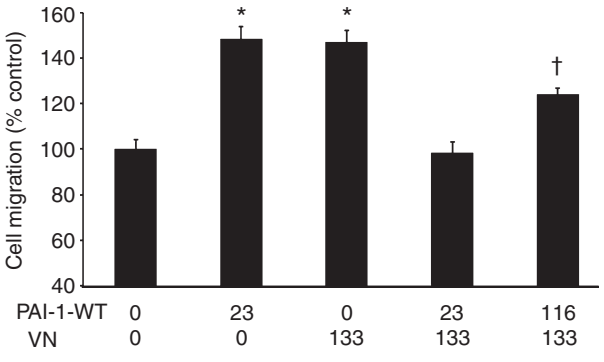

Fig. 3. Extracellular matrix (ECM) plasminogen activator inhibitor-1 (PAI-1) and vitronectin (VN) increase migration of wild-type (WT) vascular smooth muscle cell (VSMC), but inhibit each other's pro-migratory effect. *X*-axis values indicate concentrations of purified PAI-1 and VN (in nM) added in each experiment. *$P < 0.001$ vs. control (i.e. no added PAI-1 or VN); †$P < 0.002$ vs. control.

PAI-1 over-expression increases VSMC through collagen, but VN blunts this effect

Based on our findings that addition of active PAI-1 to collagen matrix promoted VSMC migration, we hypothesized that genetic over-expression of PAI-1 would also enhance VSMC migration. To test this hypothesis, we isolated aortic VSMC from PAI-1-Tg mice. SDS-PAGE/Western blot analysis of cultured PAI-1-Tg VSMC confirmed that they expressed significantly more PAI-1 than WT VSMC (Fig. 4). Consistent with these data, the PAI-1 concentration in CM of confluent PAI-1-Tg VSMC was significantly higher than that of confluent WT VSMC (430 ± 88.1 vs. 154 ± 13.3 ng PAI-1 mg^{-1} total protein, respectively, $n = 4$ per group, $P = 0.02$), whereas PAI-1 was undetectable in CM of confluent PAI-1-deficient VSMC. PAI-1-Tg VSMC migrated through collagen significantly faster than WT VSMC (Fig. 5), suggesting that over-expression of PAI-1 by VSMC, as occurs in human vascular diseases [4], promotes migration through collagen. We also compared the migration of WT and PAI-1-Tg VSMC in collagen matrices supplemented with VN (10 μg mL^{-1}). Under these conditions, the mean number of VSMC migrating through the collagen + VN matrix in 72 h was higher for PAI-1-Tg VSMC than WT VSMC, but the difference between cell types did not achieve statistical significance (Fig. 5). Furthermore, the ratio of the number of VSMC (i.e. PAI-1-Tg/WT) that migrated through a collagen-only matrix in 72 h

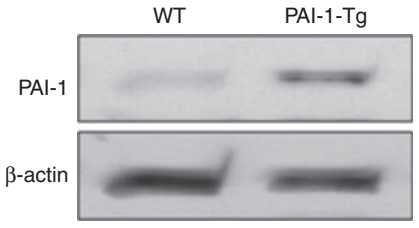

Fig. 4. Western blot analysis of plasminogen activator inhibitor-1 (PAI-1) expression by cultured vascular smooth muscle cell (VSMC). Blots were stripped and re-probed with anti-beta-actin antibody. Results shown are representative of three independent experiments.

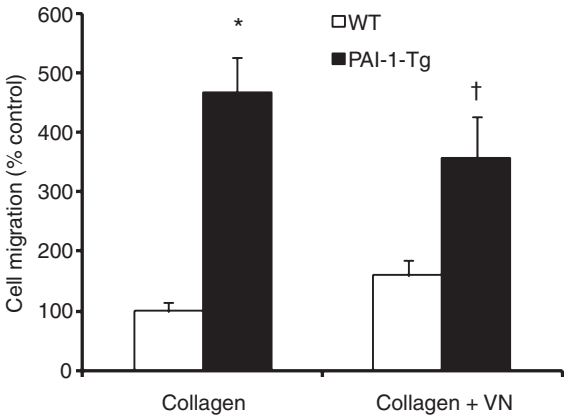

Fig. 5. Plasminogen activator inhibitor-1 (PAI-1) over-expression promotes vascular smooth muscle cell (VSMC) migration through collagen matrix, but vitronectin (VN) blunts this effect. Wild-type (WT) and PAI-1-Tg VSMC were seeded on collagen matrices and collagen matrices containing VN (collagen + VN). Migration was assessed and expressed as % control (WT VSMC migrating through collagen). $^{*}P < 0.03$ vs. WT VSMC/collagen matrix. $^{\dagger}P = 0.09$ vs. WT VSMC/collagen + VN matrix.

(4.7 ± 0.8, $n = 3$) was significantly higher ($P < 0.04$) than the ratio of PAI-1-Tg/WT VSMC that migrated through a collagen + VN matrix (2.4 ± 0.5, $n = 3$). Together, these results suggested that the pro-migratory effect of PAI-1 over-expression is blunted by VN.

Discussion

In some studies PAI-1 has been reported to inhibit VSMC migration [8], whereas in others PAI-1 has been reported to promote VSMC migration [10]. These experiments involved cell culture on two-D surfaces coated with purified VN or another ECM molecule and the addition of purified PAI-1 into the experimental system. These discordant results beg the questions, what is the net effect of PAI-1 on VSMC migration under physiological conditions reflective of the arterial wall *in vivo*, and how does PAI-1 produced by VSMC themselves (which are likely to be the dominant source of PAI-1 within the vascular media [30]) affect migration? To address these questions, we studied the migration of VSMC with variable levels of PAI-1 expression through three-D type I collagen matrices, as type I collagen is a major component of the ECM of the arterial media and intima [31]. We found that a basal level of PAI-1 expression by VSMC inhibits migration through collagen. It has been proposed that an important mechanism underlying VSMC migration is activation of plasminogen by uPA bound to its cell surface receptor, uPAR, resulting in plasmin formation and digestion of ECM by plasmin or metalloproteinases activated by plasmin. Under such a scenario, PAI-1 would be expected to inhibit cell migration by inhibiting uPA. However, we found no data to support this mechanism in our experimental system (which contained plasminogen because serum was added to culture media), as reconstitution of PAI-1-deficient VSMC with recombinant PAI-1-WT promoted, rather than inhibited, migration, whereas PAI-1-R, a mutant devoid of anti-proteolytic activity, mimicked the anti-migratory effect of endogenous PAI-1 expression. The capacity of PAI-1 to inhibit VSMC migration through collagen was critically dependent on VN expression, as demonstrated by the inability of PAI-1-R to inhibit migration of VN-deficient VSMC, and by the contrasting effects of PAI-1 deficiency in VN-expressing vs. VN-deficient VSMC (Fig. 1). VN engages integrins and uPAR on VSMC to promote adhesion and migration [32]. Given that VN binds collagen [28], our data suggest that VN secreted by VSMC bridges collagen and cell surface VN receptors to promote migration, and that PAI-1 can inhibit the migration of VSMC through collagen by blocking the bridging function of VN.

Conversely, we found that PAI-1-Tg VSMC migrate through collagen faster than WT VSMC do. The pro-migratory effect of enhanced PAI-1 expression appeared to depend on its anti-protease and LRP binding properties, as addition of latent PAI-1, PAI-1-R, or PAI-1-R76E, I91L to PAI-1-deficient VSMC did not stimulate migration, whereas addition of PAI-1-WT or PAI-1-AK did. Previous studies demonstrated that PAI-1 binds to uPA and other proteases, which results in exposure of a cryptic LRP binding site in PAI-1 [11]. Engagement of LRP by PAI-1 leads to internalization of PAI-1-protease complex, LRP, and associated integrin, thereby enabling cell detachment, which is necessary for migration [19,33]. Our results demonstrate that VSMC PAI-1 expression can stimulate migration under physiological conditions, but apparently only if PAI-1 expression level is increased, as a pro-migratory effect of PAI-1 was observed in PAI-1-Tg VSMC, but not in WT cells. PAI-1 over-expression within the vascular wall occurs in disease states characterized by migration of VSMC from the media to the intima, including diabetes mellitus and atherosclerosis [4,5]. Based upon our results we hypothesize that basal PAI-1 expression by VSMC, as occurs in a healthy artery, may inhibit intimal hyperplasia, whereas enhanced PAI-1 expression by VSMC, as occurs in diabetes mellitus and other disease states, may promote intimal hyperplasia. Consistent with this hypothesis, Otsuka *et al.* [34] observed less intimal hyperplasia in WT mice than in PAI-1-deficient mice, whereas up-regulation of arterial PAI-1 expression by TGF-beta-1 promoted intimal hyperplasia. Active PAI-1 spontaneously converts to a latent form, a process that is delayed by binding of VN to PAI-1 [35]. While Degryse *et al.* [10] observed that latent PAI-1 produced a motogenic effect in VSMC cultured on a two-D surface, we did not find any apparent effect of latent PAI-1 on VSMC migration through three-D collagen, consistent with the low affinity of latent PAI-1 for both VN and LRP [11].

Our experiments involving VN-deficient VSMC provide important insights into the role of de novo VN expression by VSMC in migration. While VN is generally considered to promote VSMC migration though interactions with integrin and non-integrin receptors on VSMC [6], we demonstrated that VN can also exert an anti-migratory effect, as WT VSMC migrated through collagen slower than VN-deficient VSMC did, addition of purified VN to collagen inhibited migration of

VN-deficient VSMC, and addition of VN to collagen matrices blunted the pro-migratory effects of recombinant PAI-1-WT and transgenic over-expression of PAI-1. However, we showed that PAI-1-deficient VSMC (which express VN) migrate faster than PAI-1/VN doubly-deficient VSMC did. Together these experiments demonstrate that the anti-migratory effect of VN depends on concomitant PAI-1 expression, whereas in the absence of PAI-1, only a pro-migratory effect of VN is observed. These results are consistent with the hypothesis that when VN expression is low, up-regulation of PAI-1 expression increases the pool of unbound, motogenic PAI-1, fostering VSMC migration. However, when VN expression is high, up-regulation of PAI-1 expression does not necessarily produce a motogenic pool of PAI-1, as PAI-1 binds VN, blocking its motogenic interactions with VSMC VN receptors, while at the same time sequestering PAI-1 from LRP [11,12]. Nevertheless, if PAI-1 expression is sufficiently high to saturate even increased levels of VN, a motogenic pool of free PAI-1 can be created. Some *in vivo* experiments involving PAI-1-knockout mice concluded that PAI-1 promotes intimal hyperplasia [36,37], others concluded that PAI-1 inhibits intimal hyperplasia [38,39], and one study found no effect [40]. While multiple factors are likely to have contributed to these variable results, it is possible that variability in VN expression between different vascular injury models [29] and mouse strains could have accounted for the variable effects of PAI-1 on neointima formation in these studies. Conversely, differences in PAI-1 expression level between experimental models may have contributed to the generation of conflicting reports that VN promotes [36] and inhibits [39] intimal hyperplasia after vascular injury.

In summary, we examined the roles of PAI-1 and VN expression by VSMC in their migration through physiological collagen matrices. Based on our results we propose the following paradigm. In isolation, VN and PAI-1 are each pro-migratory, the former by fostering cell adhesion, the latter by fostering cell detachment – both of which are necessary in coordinated fashion for VSMC migration. However, via formation of a high-affinity, non-motogenic complex, PAI-1 and VN each buffers the other's pro-migratory effect (Fig. 6). Binding of PAI-1 to VN blocks VN's interactions with its VSMC receptors, thereby inhibiting cell adhesion. Binding of VN to PAI-1 can inhibit exposure of PAI-1's LRP binding site and may sequester PAI-1 from interacting with VSMC, thereby inhibiting PAI-1's pro-migratory properties [11,12]. Hence, PAI-1 and VN exhibit a distinct functional interdependence in regulating VSMC migration [41]. The level of PAI-1 expression by VSMC and the concentration of VN in ECM are critical determinants of whether PAI-1 (or VN) promotes or inhibits migration. If VN expression is low, free PAI-1 is more likely to accumulate and stimulate VSMC migration after exposing its LRP binding site. However, if VN expression is high, PAI-1 is more likely to bind VN, thereby producing an anti-migratory effect by blocking VN's interactions with cell surface receptors.

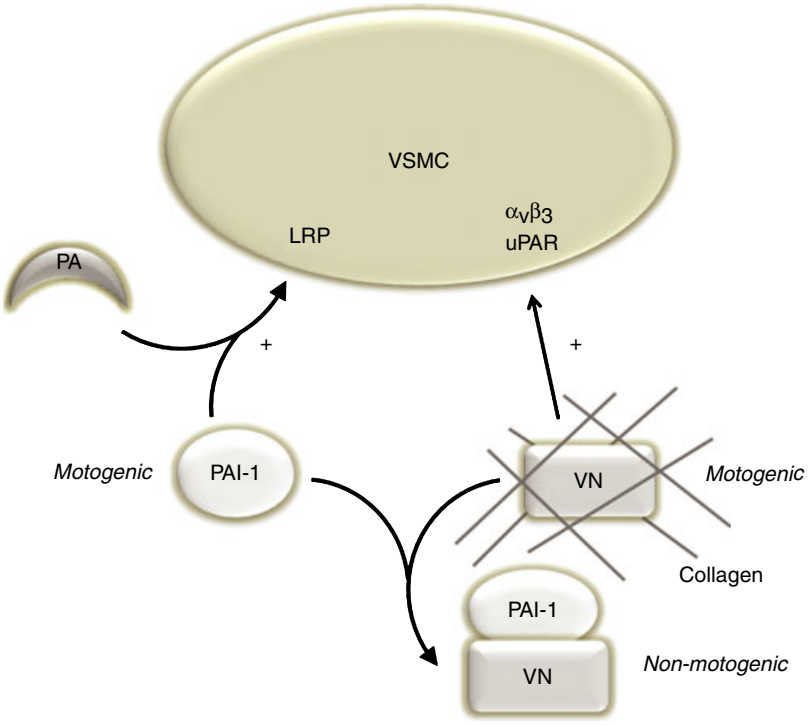

Fig. 6. Pro- and anti-migratory effects of plasminogen activator inhibitor-1 (PAI-1) and vitronectin (VN) on vascular smooth muscle cell (VSMC). VN binds collagen and stimulates (+) VSMC migration by interacting with $\alpha_v\beta_3$ and uPA receptor (uPAR). PAI-1 stimulates VSMC migration by binding to a plasminogen activator (PA), which exposes an low density lipoprotein receptor-related protein (LRP) binding site on PAI-1, leading to engagement of the complex with LRP, a motogenic receptor. Binding of PAI-1 to VN yields a complex that is not motogenic, providing a mechanism for each factor to inhibit the pro-migratory effect of the other.

Conversely, if PAI-1 expression is low, unbound VN can promote VSMC migration, whereas if PAI-1 expression is high, VN is more likely to be consumed into PAI-1-VN complex, thereby inhibiting PAI-1's pro-migratory function. This paradigm establishes the important role of PAI-1/VN stoichiometry in determining each factor's vascular effects. While our *in vitro* assay allowed us to study VSMC migration under physiological conditions, it did not reproduce several important factors present *in vivo*, including complex, multi-component ECM, cell types other than VSMC, and other factors that regulate cell migration, including thrombin, metalloproteinases, and cytokines. Therefore, animal studies will be necessary to further test the hypothesis that PAI-1/VN stoichiometry is an important regulator of arterial remodeling.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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