Smad2-Dependent Protease Nexin-1 Overexpression Differentiates Chronic Aneurysms From Acute Dissections of Human Ascending Aorta

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Objective—Tissue activation of proteolysis is involved in acute intramural rupture (dissections, acute ascending aortic dissection) and in progressive dilation (aneurysms, thoracic aneurysm of the ascending aorta) of human ascending aorta. The translational aim of this study was to characterize the regulation of antiproteolytic serpin expression in normal, aneurysmal, and dissecting aorta.

Approach and Results—We explored expression of protease nexin-1 (PN-1) and plasminogen activator inhibitor-1 and their regulation by the Smad2 signaling pathway in human tissue and cultured vascular smooth muscle cells (VSMCs) of aneurysms (thoracic aneurysm of the ascending aorta; n=46) and acute dissections (acute ascending aortic dissection; n=10) of the ascending aorta compared with healthy aortas (n=10). Both PN-1 and plasminogen activator inhibitor-1 mRNA and proteins were overexpressed in medial tissue extracts and primary VSMC cultures from thoracic aneurysm of the ascending aorta compared with acute ascending aortic dissection and controls. Transforming growth factor-β induced increased PN-1 expression in control but not in aneurysmal VSMCs. PN-1 and plasminogen activator inhibitor-1 overexpression by aneurysmal VSMCs was associated with increased Smad2 binding on their promoters and, functionally, resulted in VSMC self-protection from plasmin-induced detachment and death. This phenomenon was restricted to aneurysms and not observed in acute dissections.

Conclusions—These results demonstrate that epigenetically regulated PN-1 overexpression promotes development of an antiproteolytic VSMC phenotype and might favor progressive aneurysmal dilation, whereas absence of this counter-regulation in dissections would lead to acute wall rupture. (Arterioscler Thromb Vasc Biol. 2013;33:2222-2232.)

Key Words: epigenomics ■ fibrinolysin ■ Marfan syndrome ■ plasminogen activator inhibitor-1 ■ serpins ■ smooth muscle cell

Progressive dilation of the thoracic ascending aorta, leading to aneurysm (TAA) formation, is a chronic pathology involving an imbalance between proteolytic degradation of the extracellular matrix (ECM) and compensating biological mechanisms of resistance to proteolytic injury.1,2 In contrast, type A dissections of the ascending aorta (AAD) are acute intramural ruptures occurring often in little dilated aorta.3 TAA and AAD correspond, respectively, to a progressive or acute loss of the ability of the arterial wall to withstand the high pressure of the circulating blood. TAA and AAD share common pathogeneses, including monogenic diseases in young patients,4 association with bicuspid aortic valves,5 and degenerative forms in older patients.6 Both TAA and AAD also share common pathological signatures, including vascular smooth muscle cell (VSMC) disappearance,5,7 areas of mucoid degeneration,8 and degradation of collagen and elastic fibers.9 We recently demonstrated that blood-borne plasminogen activation is involved in the degradation of adhesive glycoproteins in TAAas and is, therefore, possibly implicated in matrix metalloprotease activation, transforming growth factor (TGF)-β release10 and VSMC disappearance.11 Similarly, markers of activation of the fibrinolytic system, including D-dimers12 and plasmin–anti-plasmin complexes,13 are biomarkers in AAD. Therefore, blood-borne proteolytic activities, outwardly convected across the arterial wall, seem to be one of the common pathways of arterial wall injury, to which

Received on: September 1, 2012; final version accepted on: June 12, 2013.
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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBBAHA.113.301327/-/DC1.
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© 2013 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBBAHA.113.301327
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VSMCs respond by applying their capacity for functional and molecular plasticity. 

Several serine protease inhibitors (serpins), including plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1), control the activity of the proteases of the plasminergic system. PN-1 is a newly recognized regulator of protease activities in the vascular wall. It is a potent inhibitor of thrombin, urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), and plasmin. PN-1 is produced and secreted by VSMCs in the arterial wall. In contrast to highly diffusible serpins like PAI-1, PN-1 is a tissue antiprotease with the remarkable property of being retained by glycosaminoglycans at the cell surface or within the ECM, thereby being barely detectable in plasma. In particular, we have shown that PN-1 protects VSMCs against plasmin-induced fibronectin degradation, cell detachment, and death.

It is of major interest that the expression of these serpins (PAI-1 and PN-1) is controlled by the TGF-β signaling pathway, which is widely assumed to play a role in aneurysmal disease, although the exact mechanisms are still subject to debate. Canonically, active plasmin releases ECM-entrapped TGF-β, which stimulates the VSMC response, including fibronectin synthesis, connective tissue growth factor secretion, antiprotease release, and proteoglycan synthesis, via, at least in part, the Smad2 signaling pathway. Therefore, despite the multiplicity of causes (including loss of function mutations of the TGF-β receptors), TGF-β pathway activation is frequently observed in TAAs. More precisely, a common event in TAA is the activation and autonomization of the Smad2 pathway, downstream to the TGF-β receptors, characterized by accumulation of activated phosphorylated-Smad2 (pSmad2) in VSMC nuclei. We recently reported that this phenomenon was controlled by the epigenetic reprogramming of the Smad2 promoter in VSMCs of the TAA wall.

Because the intramural generation of plasmin might play a major role in pathologies of the ascending aorta, including progressive dilation (TAA) and acute wall rupture (AAD), we raised the question of the biological differences between TAA and AAD and hypothesized that increased antiprotease expression could be involved. We explore here the expression of antiproteases, PN-1 and PAI-1, which are able to inhibit the activation of the fibrinolytic system in human TAA and AAD tissues and its impact on the VSMC response to pericellular plasminogen activation.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
The clinical data associated with this series of patients are presented in Table 1, and do not significantly differ from what we previously reported. Sections of aortic media from control patients showed a normal arrangement in which VSMC layers were separated by prominent elastic lamellae, interconnected by a network of small elastic fibers, among collagen fibers and proteoglycans. No atherosclerotic lesions were observed (data not shown). As compared with normal aorta (Figure 1A and A′), TAA and AAD presented common pathological features of mucoid degeneration (Figure 1B, 1B′; 1C, and 1C′). Cell death detection ELISA, or terminal deoxynucleotidyl transferase mediated UTP nick end labeling (TUNEL), revealed the absence of apoptotic cells in controls (Figure 1A′). TUNEL-positive cells were rare in TAA (Figure 1B′) and were mainly present in the vicinity of mucoid degenerative areas. TUNEL-positive cells were more frequent in dissections (AAD) where they predominated in the vicinity of the false channel (Figure 1C′).

PN-1 Overexpression in Aneurysmal But Not in Dissected Media
In control aortas, immunostaining of PN-1 was weak and diffuse, always associated with VSMCs (Figure 2A and 2A′; Figure I in the online-only Data Supplement). In TAA,
disorganization of the media was evident with the presence of areas devoid of VSMCs. PN-1 immunoreactivity was markedly increased (Figure 2B) and was still mainly associated with VSMCs as confirmed at high magnification (Figure 2B').

Degenerative mucoid areas were consistently negative for PN-1. Immunostaining of PN-1 on AAD sections was similar to that observed in controls (Figure 2C and 2C'). When analyzed by electron microscopy, PN-1 labeling was weak within the ECM and absent in the cytoplasm of normal VSMCs (not shown). In contrast, immunostaining was detected within the lumen of the Golgi apparatus, near the nucleus (Figure 2D), and in the rough endoplasmic reticulum (Figure 2E) of TAA VSMCs. Gold particles were also observed within cytoplasmic vesicles (Figure 2D, inset), associated with the cell membrane or were localized in the pericellular space associated by affinity with the ECM.

In accordance with immunohistochemical results, PN-1 mRNA (Figure 2F) and protein (Figure 2H) were found to be overexpressed in TAA compared with control aortic tissues. PN-1 was not detectable in aortic tissue–conditioned medium. Similar results were obtained for PAI-1, with an increase at both mRNA and protein levels (Figure 2G and 2I) but, in contrast, diffusible PAI-1 was easily measurable in the conditioned medium. Increased PAI-1 protein levels were observed in TAA-conditioned medium compared with that of control aorta (Figure 2J). PN-1 and PAI-1 overexpression was equivalent, irrespective of the cause of the aneurysmal disease (data not shown).
We confirmed that mRNA expression of both t-PA and u-PA was similarly significantly increased in TAA (1.41±0.41 and 2.94±0.56 AU, respectively)\textsuperscript{11} and AAD (1.18±0.11 and 2.45±0.22 AU, respectively) as compared with controls (0.22±0.11 and 0.86±0.26, respectively; \(P<0.01\)), whereas PN-1 was specifically overexpressed in TAA (0.29±0.04 AU), but not in AAD (0.05±0.01 AU) as compared with controls (0.10±0.03 AU; \(P=0.1\) versus AAD, \(P<0.001\) versus TAA). Then, we quantified the expression of these factors of the plasminergic system in primary cultures of control and TAA VSMCs in which we assessed the expression of the canonical SM marker genes SM\(\alpha\)A, SM22 and myosin, heavy chain 11 (Figure II in the online-only Data Supplement). Staining for these SM marker genes confirmed that our in vitro model is a homogeneous population of VSMCs. In contrast with tissue extracts, in VSMC cultures, there was no difference in expression of t-PA (control: 0.24±0.11 AU versus TAA: 0.20±0.10 AU; NS) or u-PA (control: 0.14±0.12 AU versus TAA: 0.11±0.07 AU; NS) between control and TAA VSMCs. TGF-\(\beta\) did not modify t-PA and u-PA expression in VSMCs of either TAA or control origin (not shown). However, in AAD tissue, although expression of t-PA and u-PA was similarly increased as compared with controls, the expression of PN-1 (Figure 3A) and Smad2 (in aortic tissue extract or cultured VSMCs; Figure 3A) remained unchanged compared with controls (\(P=0.9\) and \(P=0.5\), respectively), and AAD thus differed significantly from TAA (\(P<0.001\) and \(P<0.001\), respectively). Quantification of positive pSmad2 nuclei in aortic tissue confirmed the mRNA expression data, showing a highly significant increase in pSmad2-positive nuclei restricted to TAA\textsuperscript{27,28} (51.30±2.50%), as compared with controls (7.25±3.01%) and AAD (6.37±6%; \(P<0.001\); Figure 3B). There was no difference in pSmad2-positive nuclear numbers between pathogeneses in TAA group (Marfan, 47.62±7.75%; bicuspidy, 52.18±6.06%; degenerative, 54.18±2.81%; NS). Similarly, the proportion of pSmad2-positive nuclei was significantly increased.

![Figure 3](https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.115.300606)  

**Figure 3.** Thoracic aneurysm of the ascending aorta (TAA) and acute ascending aortic dissection (AAD) do not share the same expression pattern. A, Protease nexin-1 (PN-1) was specifically overexpressed in TAA (\(P<0.001\)), but not in AAD (\(P=0.1\) as compared with controls, and \(P<0.001\) as compared with TAA). In tissue extracts, Smad2 mRNA levels were significantly increased in TAA as compared with controls and dissections (top), and this overexpression was conserved in primary vascular smooth muscle cell (VSMC) cultures (bottom; aortic tissue extracts, TAA: \(n=12\); AAD: \(n=5\); and control: \(n=10\) and SMC cultures, TAA: \(n=8\); AAD: \(n=5\); and control: \(n=6\)). B, Phosphorylated-Smad2 (pSmad2) immunostaining was very low in controls as compared with TAAs, irrespective of the cause. The numbers of pSmad2-positive nuclei were similar in AAD and controls and, therefore, very different from those of TAA (magnification, \(x40\); hematoxylin counterstaining. TAA: \(n=41\); AAD: \(n=25\); and control: \(n=33\) fields). These tissue data were confirmed in primary VSMC cultures showing a significant increase in pSmad2-positive nuclei in TAA VSMC as compared with AAD and control VSMC (\(P<0.001, n=33\) for TAA; \(n=43\) for AAD; and \(n=47\) fields for controls, in 5 primary cultures for each condition). C, In contrast, there is no change in total transforming growth factor-\(\beta\) (TGF-\(\beta\)) secretion between groups. \(*P<0.05\) and \(**P<0.001.\)
in cultured VSMCs of TAA origin (81.09±14.65%) as compared with those of control or AAD origin (39.57±30.38%, 42.45±31.46%, respectively; \( P<0.001 \); Figure 3B).

In contrast with PN-1, PAI-1 mRNA expression (control, 0.12±0.05 AU; TAA, 0.31±0.08 AU; and AAD, 0.25±0.07 AU) was increased both in TAA and in AAD compared with controls (\( P<0.001 \) and \( P<0.01 \), respectively), and thus did not significantly differ between TAA and AAD (\( P=0.1 \)).

**PN-1 and PAI-1 Overexpression in VSMCs From TAA Is Dependent on Smad2 activation**

In control VSMCs, treatment with TGF-\( \beta \)-1 increased PN-1 and PAI-1 mRNA (Figure 4A and 4B). It is of major interest that, in TAA VSMCs, basal levels of PN-1 and PAI-1 were increased before stimulation compared with controls, indicating a constitutive overexpression of these serpins (\( P<0.05 \)) and TGF-\( \beta \)-1 addition did not induce any further increase in PN-1 expression. In these culture conditions, the active form of TGF-\( \beta \)-1 was not detectable in serum-deprived culture media of VSMCs, originating from control, aneurysmal or dissecting aorta. Moreover, total TGF-\( \beta \)-1 after acidification of culture media did not differ between control, aneurysmal and dissecting aortic VSMCs, or between VSMCs from TAA of the different pathogeneases (controls, 272.60±180.90; TAA Marfan, 461.80±250.30; bicuspid aortic valves, 583.90±324.50; degenerative, 243.20±82.01; dissection, 261.50±116.90 pg/mL; NS; Figure 3B). In parallel, TGF-\( \beta \)-1 mRNA expression was not significantly modified between VSMCs of TAA origin as compared with those from controls (TAA VSMCs, 0.65±0.39 versus 0.57±0.37 AU in control VSMCs; NS). Therefore, we can conclude that the constitutive overexpression of PN-1 in VSMCs of TAA origin is independent of TGF-\( \beta \)-1.

In contrast, PAI-1 expression in aneurysmal cells was still sensitive to TGF-\( \beta \)-1 but the induced increase was significantly

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**Figure 4.** Transforming growth factor-\( \beta \)-1 (TGF-\( \beta \)-1)–induced serpin overexpression is dependent on Smad2. **A**, Regulation of protease nexin-1 (PN-1) by TGF-\( \beta \)-1 and thrombin receptor-activating peptide (TRAP). Basal levels of PN-1 were significantly higher in thoracic aneurysm of the ascending aorta (TAA)–derived than in control vascular smooth muscle cells (VSMCs; TAA: 1.14±0.12 vs control: 0.59±0.07 AU; TAA Marfan, 1.74±0.42 vs control: 0.91±0.34 AU; TAA Marfan, 1.92±0.36 vs control: 1.14±0.24 AU; TAA bicuspid aortic valves, 1.51±0.28 vs control: 0.82±0.12 AU; TAA degenerative, 1.27±0.21 vs control: 0.60±0.15 AU). Exposure to TGF-\( \beta \)-1 (5 ng/mL, 24 hours) induced an increase in PN-1 expression in control but not in TAA-derived VSMCs (\( * P<0.01 \)) and TGF-\( \beta \)-1 addition did not induce any further increase in PN-1 expression. In these culture conditions, the active form of TGF-\( \beta \)-1 was not detectable in serum-deprived culture media of VSMCs, originating from control, aneurysmal or dissecting aorta. Moreover, total TGF-\( \beta \)-1 after acidification of culture media did not differ between control, aneurysmal and dissecting aortic VSMCs, or between VSMCs from TAA of the different pathogeneases (controls, 272.60±180.90; TAA Marfan, 461.80±250.30; bicuspid aortic valves, 583.90±324.50; degenerative, 243.20±82.01; dissection, 261.50±116.90 pg/mL; NS; Figure 3B). In parallel, TGF-\( \beta \)-1 mRNA expression was not significantly modified between VSMCs of TAA origin as compared with those from controls (TAA VSMCs, 0.65±0.39 versus 0.57±0.37 AU in control VSMCs; NS). Therefore, we can conclude that the constitutive overexpression of PN-1 in VSMCs of TAA origin is independent of TGF-\( \beta \)-1.

In contrast, PAI-1 expression in aneurysmal cells was still sensitive to TGF-\( \beta \)-1 but the induced increase was significantly
less important in aneurysmal VSMCs than in controls. To determine whether the increased serpin expression was specific to the TGF-β1 pathway activation, we stimulated VSMCs with thrombin receptor-activating peptide known to differentially regulate the expression of PN-1 (downregulation) and PAI-1 (upregulation), and to act independently of the Smad pathway (mitogen-activated protein kinases). Incubation with thrombin receptor-activating peptide induced downregulation of PN-1 and upregulation of PAI-1 expression, similarly in control and aneurysmal VSMCs (Figure 4A and 4B). To further investigate the participation of Smad2 in the regulation of serpin expression, we performed transfection of small interfering RNA targeting Smad2 in TAA or control VSMCs and this resulted in a significant decrease in PN-1 expression (Figure 4C). Expression of PN-1 and PAI-1 induced by TGF-β1 was also blocked by Smad2 knockdown (Figure 4B and 4C) providing compelling evidence of the involvement of Smad2 in the TGF-β1–induced regulation of PN-1 and PAI-1 expression in normal VSMCs.

**PN-1 Overexpression in TAA Is Associated With the Recruitment of Smad2 on Its Promoter**

We have previously observed and confirmed here (Figure 3; Figure III in the online-only Data Supplement) an autonomous overexpression of Smad2 and its constitutive phosphorylation and activation in aneurysmal VSMCs.28 Because we demonstrated that PN-1 and PAI-1 expression were both dependent on the Smad2 pathway, the expression profile of the 2 serpins in TAA highly suggests that their constitutive overexpression could be because of the increased recruitment of Smad2 on the PN-1 and PAI-1 promoters. Indeed, in silico analyses of the PN-1 and PAI-1 promoters revealed the presence of 1 putative Smad binding site on the PN-1 promoter (for sequences and localizations; Figure 5A and 5B) and 5 Smad consensus binding sites were identified on the PAI-1 promoter. Chromatin immunoprecipitation assays using an anti-Smad2 antibody showed a significant increase in the Smad2 enrichment on the PN-1 promoter in TAA aortic tissues (Figure 5C). Using several sets of primers targeting the total PN-1 promoter length between −1000 and +200 base pairs, we observed that Smad2 enrichment is strictly restricted to the region of the PN-1 promoter containing the Smad consensus binding site. Similarly, an increase in Smad2 enrichment was also observed on the PAI-1 promoter in TAA aorta as compared with control aorta (Figure 5D). These results provide evidence of a correlation between the increased expression of PN-1 and PAI-1 in TAA and the recruitment of Smad2 on their promoters.

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**Figure 5.** Smad2 enrichment on the protease nexin-1 (PN-1) and plasminogen activator inhibitor-1 (PAI-1) promoters of thoracic aneurysm of the ascending aorta (TAA) vascular smooth muscle cells (VSMCs). Table (A) and schematic representations (B) of the localization of putative Smad binding sites (blue boxes) on PN-1 and PAI-1 promoters upstream to the transcription starting site (TSS, arrow), C. Smad2 enrichment on the PN-1 promoter. Results are expressed as the ratio of the immunoprecipitated sample (IP) value/the nonimmunoprecipitated sample value (INPUT). A significant enrichment of Smad2 on the PN-1 promoter was detected in TAA aortas (position −800/−1000; TAA: 0.8±0.06 vs control: 0.24±0.031; P<0.001 and position −600/−800; TAA 0.73±0.08 vs control: 0.2±0.02; P<0.005). The curves represent Smad2 binding on the PN-1 promoter (−1000/+200 base pairs) in TAA and control aortic tissues. D. A significant increase in Smad2 enrichment was detected on the PAI-1 promoter in TAA compared with control aortas (position −1000/−800 base pairs; TAA: 0.8±0.06 vs control: 0.24±0.031; P<0.0001 and position −800/−600 base pairs; TAA: 0.72±0.08 vs control: 0.2±0.02; P<0.001). The curves are representative of Smad2 binding on the PAI-1 promoter (−1000/+200 base pairs) in TAA and control aortic tissues. A-D, TAA: n=9 (Marfan, n=3; degenerative, n=3; and bicuspid aortic valves [BAV], n=3) and control (n=5). *P<0.05.
Effect of PN-1 and PAI-1 Overexpression on Plasmin-Induced Apoptosis in TAA VSMCs

To investigate the effect of the Smad-dependent constitutive overexpression of PN-1 and PAI-1 in VSMCs from TAA, we analyzed plasminogen activation–induced VSMC detachment. Incubation with plasmin or plasminogen (zymogen) is known to induce anoikis in VSMCs, a process consisting of apoptosis caused by cell detachment.\(^{21}\) Pericellular inhibition of plasmin or plasminogen activation was assessed by different methods. Plasminogen activation was first analyzed on the surface of VSMCs that were pretreated or not by TGF-\(\beta\)-1 for 48 hours. In normal VSMCs, TGF-\(\beta\)-1 treatment induced a dramatic decrease in plasmin generation, regardless of the TGF-\(\beta\)-1 concentration used (Figure 6A). This effect was partially reversed by addition of antibodies blocking PN-1 or PAI-1, providing evidence that TGF-\(\beta\)-1–induced PN-1 and PAI-1 overexpression counteracts plasminogen activation in VSMCs. We checked that no changes in mRNA levels of the plasminogen activators, t-PA and u-PA, occurred in response to TGF-\(\beta\)-1 under the same experimental conditions (data not shown), showing that the effect of TGF-\(\beta\)-1 on VSMC plasminogen activation mainly depends on serpin expression. In nontreated-aneurysmal VSMCs, the basal level of plasmin generation was significantly lower than that of control cells (\(P<0.01\)), and TGF-\(\beta\)-1 treatment resulted in a further decrease in plasminogen activation (Figure 6A). Incubating plasmin (0.5 nmol/L) in the presence of normal VSMCs resulted in a decrease in plasmin activity as compared with the absence of cells (Figure 6B). This inhibition was increased when the VSMCs were pretreated with TGF-\(\beta\)-1 and decreased in the presence of PN-1–blocking antibodies, demonstrating a role of PN-1 in this pericellular plasmin inhibition.

We then investigated the effect of plasminogen on control and aneurysmal aortic VSMCs in culture (Figure 6C). Direct incubation of plasminogen at both concentrations (500 and 1000 nmol/L) induced a significant decrease in adherence of normal VSMCs in culture, whereas no detachment was observed for TAA-derived VSMCs. These results correlated with the plasmin activity present in the VSMC supernatant.

![Figure 6](image-url)

**Figure 6.** Plasminogen activation in control and thoracic aneurysm of the ascending aorta (TAA)–derived smooth muscle cells (SMCs). A, Vascular SMCs (VSMCs) incubated with plasminogen. Normal and TAA-derived VSMCs were first incubated with transforming growth factor (TGF)-\(\beta\)-1 (5 ng/mL) for 48 hours before treatment with plasminogen. Plasminogen activation was strongly inhibited by TGF-\(\beta\)-1 treatment in normal VSMCs. In nontreated-aneurysmal VSMCs, the basal level of plasmin generation was significantly lower than that of control VSMCs. In the TAA cells, TGF-\(\beta\)-1 stimulation induced a further decrease in the generation of plasmin activity. Incubation of the cells with antibodies blocking protease nexin-1 (PN-1) and plasminogen activator inhibitor-1 (PAI-1) resulted in increased plasminogen activation. No effect was observed with an irrelevant antibody (not shown). B, Normal VSMCs were stimulated or not with TGF-\(\beta\)-1 and incubated with plasmin (0.5 nmol/L). Plasmin activity was significantly inhibited by control cells as compared with the absence of cells, and further decreased when the VSMCs were pretreated with TGF-\(\beta\)-1. The PN-1–blocking antibody partially restored plasmin activity, whereas no effect was observed with an irrelevant antibody (not shown). C, Normal or TAA-derived VSMCs were treated with or without plasminogen for 48 hours and the number of adherent cells was then assayed by colorimetry. Triplicate assays were performed for each condition, and results were expressed relative to the untreated control. Error bars: SEM values obtained after grouping results from 3 independent experiments. Plasminogen treatment induced detachment of normal VSMCs, whereas no detachment was observed for TAA-derived VSMCs (*\(P<0.05\)) at either plasminogen dose (\(n=3\times3\) for each condition).
In contrast to normal VSMCs, no significant plasmin activity could be measured in TAA-derived VSMC supernatant, indicating that pericellular plasmin generation and subsequent hydrolysis of adhesive contacts mediated cell detachment.

The resistance to apoptosis was visualized using TUNEL staining. Exposure to plasmin induced an increase in TUNEL staining in normal VSMCs (Figure 7A). In contrast, no difference in staining was observed in aneurysmal VSMCs exposed or not to 10 mmol/L plasmin (Figure 7B). Incubation with a PN-1 blocking antibody abolished the resistance of aneurysmal VSMCs to plasmin-induced apoptosis and cell detachment, as demonstrated by the absence of VSMCs (4',6-diamidino-2-phenylindole and TUNEL staining; Figure 7C). In contrast, neutralization of PAI-1 by a specific antibody had a limited effect on control and aneurysmal VSMCs (data not shown). PAI-1 thus seems to be a more dispensable factor compared with PN-1 in the protection against plasmin-induced apoptosis.

Discussion

We previously reported that the fibrinolytic system is activated in vitro in contact with VSMCs and in vivo in the arterial wall of chronic aneurysm of the ascending aorta. Unfortunately in this study, plasmin/plasminogen could not be reliably measured in samples of dissected aorta because of massive contamination by blood clotting and fibrinolysis in the false lumen. Moreover, risk factors are not entirely the same between TAA and AAD, differences that could influence the results. However, we were able to show overexpression of the plasminogen activators t-PA and u-PA in AAD, as in TAA, where such overexpression leads to an increased production of active plasmin in the aneurysmal wall. In parallel, we report that the constitutive overexpression and activation of Smad2 in TAA, which escapes TGF-β1 control, were not observed in AAD at the tissue or cell level. PAI-1 and PN-1 are 2 serpins able to block the plasminergic system at different levels. Both are synthesized by VSMCs but they differ with respect to their physiology; compared with the diffusible PAI-1, readily detectable in plasma, PN-1 is mainly an intratissue serpin, associated with extracellular and intracellular proteoglycans and undetectable in plasma. PN-1 is also highly expressed and secreted by activated platelets and thrombus formation, a phenomenon which takes place in the false channel of AAD and thus could potentially contaminate the arterial wall at the protein level. There is general agreement that PAI-1 is protective against aneurysm development because of its antiprotease properties. Indeed, PAI-1 overexpression prevented the formation of experimental aneurysms, and PAI-1 expression is decreased in the aneurysmal abdominal aorta wall as compared with control. In contrast, the expression and role of PN-1 have never been explored in aneurysmal diseases. We observed that the serpins, PAI-1 and PN-1, are chronically and constitutively overexpressed under the control of Smad2 in aneurysms of the ascending aorta but not in dissections. Because Smad pathway activation induces synthesis and secretion of ECM components, including collagens, tropoelastin, fibronectin, proteoglycans, and antiproteases by VSMCs, it could be considered as a compensatory response to proteolytic injury, limiting damage and permitting chronic progressive evolution of dilatation rather than acute rupture. Indeed, such a compensatory phenomenon seems to be absent in acute dissection.

The first observation of the present study is the importance of tissue PN-1 expression in the TAA wall compared with dissected and normal arterial wall and its close association with VSMCs, as demonstrated by immunohistochemistry, electron microscopy, and Western blot. This phenomenon could be, at least in part, because of the increases in PN-1 synthesis, but PN-1–protease complexes are actively removed from the tissue.

**Figure 7.** A, Plasmin-induced vascular smooth muscle cell (VSMC) detachment and apoptosis. B, Control and aneurysmal VSMCs were incubated with plasmin in presence or absence of transforming growth factor (TGF)-β1 and protease nexin-1 (PN-1) neutralizing antibody (Ab PN-1). Exposure to plasmin-induced apoptosis in control VSMCs (TUNEL-positive cells). In contrast, plasmin had no effect on aneurysmal VSMCs which remained negative for TUNEL staining. C, Addition of TGF-β1 abolished the effect of plasmin in control VSMC cultures (TUNEL). The role of PN-1 in the inhibition of plasmin-induced VSMC apoptosis was investigated by incubation with a PN-1 neutralizing antibody. The neutralization of PN-1 enhanced the effect of exposure to plasmin and induced cell detachment in control, as well as in aneurysmal VSMCs. It also totally abolished the protective effect of TGF-β1 in control VSMCs (n=3×3 for each condition).
extracellular environment by internalization via the low-density lipoprotein receptor–related protein 1, dependent or not on glycosaminoglycans. Therefore, the observed increase in tissue PN-1 could also be, in part, because of an increased clearance and an intracellular retention of PN-1–protease complexes. This endocytosis of PN-1 complexes by VSMCs probably explains our observations in electron microscopy: extracellular PN-1 in controls, extra- and intracellular PN-1 in TAAs. The latter was observed not only in the Golgi apparatus (increased synthesis) but also in intracytoplasmic vesicles.

Although the expression of PN-1 in renal and neural cells is known to be regulated by TGF-β1, the effect of TGF-β1 on PN-1 expression in human VSMCs and the exact role of the Smad2 pathway have not been previously explored as extensively as for PAI-1 expression. Here, we show that PN-1 expression in VSMCs is associated with the recruitment of Smad2. The dramatic effect of Smad2 knockdown on PN-1 expression provides compelling evidence for a major role of Smad2 in the regulation of PN-1 expression in aneurysmal VSMCs, and the epigenetic reprogramming of the Smad2 promoter previously described, leading to constitutive Smad2 overexpression and activation in TAA, probably explains this observation. In contrast, the absence of overexpression and activation of Smad2 in AAD tissue and VSMCs is consistent with the lack of PN-1 overexpression in this pathology.

Our results show an increase in both mRNA and PAI-1 protein levels in TAA and of mRNA in AAD. The mitogen-activated protein kinases pathway in TGF-β1–stimulated SMCs can also induce PAI-1 expression. This noncanonical TGF-β signaling pathway could account for the limited increase in PAI-1 expression observed in response to TGF-β treatment. The overexpression of PAI-1 mRNA in AAD could also be related to its postactivation de novo synthesis by platelets, which are highly activated and aggregated in the false lumen of dissected aortic walls.

The activation of Smad2 is now well characterized in aneurysms of the ascending aorta. It is a common manifestation in all types of TAA, both syndromic and nonsyndromic. This activation is associated with a constitutive increase in Smad2 expression in VSMCs. Smad2 expression is controlled by an epigenetic mechanism consisting of a modification in the histone code on the Smad2 promoter. The epigenetic regulation of Smad2 is characterized by its constitutive (TGF-β1 independent), heritable (conserved through mitosis), and VSMC-specific properties. The expression and activation of Smad2 potentially cause important changes in the VSMC expression pattern leading to a modification of VSMC phenotype.

<table>
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<th>Table. Clinical Characteristics of Patients</th>
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<td>AAD indicates acute ascending aortic dissection; ACEI, angiotensin-converting enzyme inhibitor; BAV, bicuspid aortic valve; and TAA, thoracic aneurysm of the ascending aorta.</td>
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<td>*P&lt;0.05, significantly different between TAA and AAD (aneurysm and dissection, respectively).</td>
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<td>$P&lt;0.05, significantly different between pathogeneses.</td>
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AAD indicates acute ascending aortic dissection; ACEI, angiotensin-converting enzyme inhibitor; BAV, bicuspid aortic valve; and TAA, thoracic aneurysm of the ascending aorta.
VSMCs possess plasticity, which allows them to modulate their phenotype in response to changes in environmental cues, such as mechanical stress, matrix integrity, and cell–matrix interactions. Importantly, we show in this study that the VSMC reprogramming, which occurs during aneurysmal development and progression, leads to the expression of serpins. Increased serpin expression in aneurysmal VSMCs constitutes a protective and antiapoptotic phenotype in vitro. Our data argue against the current idea of a unique deleterious effect of Smad2 activation in TAA, suggesting that Smad2 activation could be a self-protective response developed by VSMCs during aneurysmal development.

In conclusion, this study has shown, for the first time, that PN-1 overexpression is one of the consequences of the epigenetic reprogramming of Smad2, which induces its overexpression and activation in human aortic VSMCs from TAA media as compared with controls and AAD. These phenomena seem to represent a response to the disease process (initial dilation and a change in mechanotransduction) rather than being involved in the pathogenesis of the disease. In contrast, overexpression of plasminogen activators occurs in both TAA and AAD and is not dependent on the epigenetic regulation of Smad2. Therefore in TAA, the VSMC reprogramming response involves PN-1 overexpression, leading to antiplasmin activity, and clearance of protease/antiprotease complexes by VSMCs, probably limiting the risk of acute events by privileging a chronic progressive action of proteases. In contrast, when these responsive processes do not take place in VSMCs, the risk of acute dissection is enhanced.

Sources of Funding
This study was supported by the French National Research Agency (BSVE1: GDPM2, and NEX-STARWALL) and the EU FP-7 integrated project Fighting Aneurysmal Disease (www.fighting-aneurysm.org/). D. Gomez was supported by a grant from the Medical Research Foundation.

Disclosures
None.

References

Aneurysms and dissections of the human ascending aorta correspond to progressive or acute loss of the ability of the arterial wall to withstand arterial blood pressure. In both diseases, blood-borne proteases, such as plasmin, play an important role, irrespective of the cause of disease. The present study shows that overexpression of the antiprotease PN-1 in aortic tissue is a consequence of the epigenetic reprogramming of Smad2 expression, inducing its overexpression and activation in human aortic vascular smooth muscle cells from aneurysmal medias but not in controls and dissections. This reprogramming probably limits the risk of acute events by privileging a chronic progressive action of proteases.
Smad2-Dependent Protease Nexin-1 Overexpression Differentiates Chronic Aneurysms From Acute Dissections of Human Ascending Aorta

Delphine Gomez, Keto Kessler, Luciano F. Borges, Benjamin Richard, Ziad Touat, Véronique Ollivier, Silvana Mansilla, Marie-Christine Bouthon, Soleyman Alkoder, Patrick Nataf, Martine Jandrot-Perrus, Guillaume Jondeau, Roger Vranckx and Jean-Baptiste Michel

*Arterioscler Thromb Vasc Biol.* 2013;33:2222-2232; originally published online June 27, 2013; doi: 10.1161/ATVBAHA.113.301327

*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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**Patients and aortic specimens**

The clinical research protocol was approved by the local ethics committee (CPP Ambroise Paré, Boulogne, France, in March 2008). All patients provided signed informed consent. Tissue specimens of aneurysms (TAA) and dissections (AAD) were collected during aortic surgery. Forty-six tissue specimens of TAA were divided into three groups according to patients’ clinical features and genetic background: 1- Marfan syndrome related to mutations in FBN1 (n=15), 2- TAA associated with bicuspid aortic valve (BAV) (n=16) and 3- degenerative ascending aortic aneurysm (n=15). Ten AAD specimens were collected in similar conditions (2 BAVs, 8 degenerative cases). However, due to the blood clotting in the false lumen, measurement of plasmin activity in these samples was not relevant to events prior to the acute dissection. Normal ascending aortas were obtained from organ transplant donors (controls, n=10) with the authorization of the French Biomedicine Agency (PFS09-007). The clinical data associated with this series of patients are presented in Table 1, and do not significantly differ from what we previously reported1. TAA tissues were sampled, from the most dilated part of the ascending aorta: the outer curvature of the aortic root in BAV and degenerative cases, the Valsalva sinus for Marfan cases. Aortic tissue preparation consisted of an immediate dissection to separate medial and adventitial layers followed by either direct freezing or enzymatic digestion2 for VSMC primary cultures (see supplemental files).

**Aortic tissue extracts**

Extractions (protein, mRNA and chromatin) were performed directly from frozen aortic media. Aortic samples were first cryogenically pulverized in liquid nitrogen, using a freezer mill. About 100 to 300 mg of crushed aortic powder was subsequently used for immunoblotting, RT-PCR, or Chromatin Immunoprecipitation (ChIP). The number of medial tissue extracts were: TAA: n=12; AAD: n=5; and control: n=10.

**VSMC primary cultures**

Medial samples of aneurysmal and normal aortas were incubated in collagenase and elastase to obtain primary cultures of aortic VSMCs2. Cells were routinely cultured in SMC medium containing 5% foetal calf serum (FCS). Passages 3 to 6 were used for experiments. Stimulations with TGF-β1 or TRAP were performed after 24h incubation in serum-free medium. Control VSMCs were incubated with TGF-β1 (Transforming Growth Factor-β1 (Sigma Aldrich)) 5ng/mL or TRAP (Thrombin Receptor-Activating Peptide), 100µM for 24 or 48 hours.

Concentrations of TGF-β1 were determined in the VSMC culture medium using ELISA kits (R&D systems) following the manufacturers’ instructions. TGF-β1 essays were performed, in parallel, on acidified (HCl, 1.5 N) and non-acidified samples as previously described3. Sample acidification permits the quantification of the total amount of TGF-β1 (latent and active forms) whereas the use of non-acidified samples permits the measurement of only active TGF-β1.

**Immunohistochemistry**

Immunohistochemistry was carried out as previously described4. The primary monoclonal antibody directed against human PN-1 (1F6) was home-made4. All slides were incubated overnight with the
primary antibody (5 µg/ml in TBS/TC solution), followed by the secondary antibody (Kit LSAB-DAKO) for 30 minutes. Similarly, slides were also incubated overnight at 4°C with anti-pSmad2 polyclonal antibody (0.5µg/ml; Chemicon). The binding reaction was detected using DAB. Slides were counterstained with Mayer’s haematoxylin. Negative control immunohistochemical procedures consisted of omission of the primary antibody from the staining protocol and primary antibody substitution by equivalent amounts of naive mouse and/or rabbit IgG. These procedures gave consistently negative results. Sections of pathological aortic tissue were compared to normal aortas. The number of cells with nuclei positive for pSmad2 within medial aortic tissue sections was obtained by counting five fields per section in 15 TAA (5 for each etiology), 10 AAD and 10 controls. Data are expressed as the percentage of total nuclei, which were pSmad2-positive as previously described. Similarly the number of cultured VSMC pSmad2 positive nuclei was obtained by counting respectively 33, 43 and 47 wells per cultures from 4 TAA, 3 AAD and 4 controls (passages 3 to 6).

VSMCs in primary cultures were characterized using functional markers: SM a-actin (Dako, 10mg/ml), SM-22a (transgelin, Abcam, 10mg/ml), MYH-11 (SM specific myosin, Dako, 10mg/ml) (supplemental figure). The number of patients examined was 3 for each condition (TAAs, AADs and controls).

**Immunoelectron microscopy**

For immunoelectron microscopy, small pieces of tissue were fixed in 3% glutaraldehyde dissolved in 0.15 M phosphate buffer at pH 7.2 for 1 hour, followed by postfixation in 1% osmium tetroxide dissolved in 0.9% sodium chloride for 1 hour. Fixed material was stained overnight with 0.5% aqueous uranyl acetate. The samples were dehydrated in a graded acetone series, and embedded in Araldite resin. Ultrathin sections, at 70 nm, were obtained using an LKB ultramicrotome, equipped with a diamond knife and placed on formvar-coated, 200-mesh, nickel grids. Sections were treated with 0.5 M Sodium Periodate in aqueous solution for 15 minutes, incubated overnight with the primary polyclonal antibody at a dilution of 5µg/ml for PN-1 (Santa Cruz) in TBS/TC solution and then incubated in a solution containing the goldconjugate (bridging antibody RuG/M. DAKO) for 2 hours. Sections were double-stained with uranyl acetate and lead citrate. The grids were studied and micrographed in a Philips TECHNAI 12 transmission electron microscope operating at 80 kV.

**Immunoblotting**

Cryogenically pulverized aortic medial tissues were homogenized in a lysis buffer (50 mM TRIS [pH 8], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) containing a cocktail of protease inhibitors and of serine/threonine and tyrosine phosphatase inhibitors (Sigma Aldrich). The protein concentration from each sample was determined (Thermo Fisher Scientific). Extracts were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5 % BSA-TBS-T (Tris-buffered saline [pH 7.4]-0.1 % Tween 20) for 1 hour. Membranes were incubated overnight (4°C) with primary antibodies to PAI-1 (Santa Cruz), PN-1 or GAPDH (1.6 µg/mL, Biovalley), washed with TBS-T and then incubated with peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat IgG (Jackson Laboratories) for 1 hour. The signal was detected using a chemiluminescence kit (ECL+ kit; Amersham). Positive immunoreactive bands were quantified by densitometry.

**Reverse Transcription - PCR**

Total RNA was extracted from frozen samples of aortic media (TAA: n=12; AAD: n=5; and control: n=10) or cultured SMCs (TAA: n=8; Control: n=6) using the EZNA kit (Omega Biotek), according to the manufacturer’s directions. Reverse Transcription was performed using kits from Invitrogen (Carlsbad) and real-time PCR in the LightCycler system with SYBR Green detection (Roche Applied Science). The mRNA levels were normalized to GAPDH mRNA.
**ChIP assay**

ChIP assay was performed as previously described on aneurysmal (Marfan: n=3; degenerative: n=3; BAV: n=3) and control (n=5) frozen media. Tissue homogenates were cross-linked with 1% formaldehyde and neutralized by addition of 125mM glycine. Chromatin isolation was performed using Magna ChIP G or Magna ChIP A (Millipore), following the manufacturer’s instructions. Immunoprecipitation (IP) was performed with an anti-Smad2 antibody (2µg/IP, Santa Cruz). DNA from non-immunoprecipitated sheared chromatin (INPUT) was also purified. PCR was then performed using specific primers. Results are expressed as the ratio IP/INPUT for each primer set.

**Smad 2 siRNA knockdown**

Control VSMCs (n=3) were transfected with 0.5 µM Accell pool small interfering RNAs (siRNAs) (Dharmacon) in Accell delivery media (B-005000). Accell siRNAs were: Accell pool Non-Targeting siRNA (D-001910-10), Accell Red Cyclophilin B siRNA as positive control (D-001975-01-20) (data not shown) and Accell pool siRNA Smad2 (E-003561-00-0020, human Smad2, 4087). 72h after transfection, proteins and RNAs were extracted using Trizol, according to the manufacturer’s instructions.

**Plasminogen activation and plasmin assays**

Control and aneurysmal VSMCs were stimulated by 5ng/ml TGF-β1 for 48 hours and then incubated for 30 min at 37°C in presence or absence of PN-1 and/or PAI-1 blocking antibodies (150 µg/ml). Human plasminogen (500 nM) or plasmin (0.5 nM), were added (30 min at 37°C) and plasmin activity was estimated by monitoring the hydrolysis of a specific fluorescent substrate during 20 hours.

**Plasmin/plasminogen-induced apoptosis assay**

Control and aneurysmal VSMCs were grown to confluence and then starved in serum-free VSMC medium. Human plasminogen (500 nM or 1000 nM) was added and VSMC detachment was estimated after 48 hours by a colorimetric method (MTT test). Plasmin activity was measured in the supernatant by monitoring the hydrolysis of a specific fluorescent substrate.

VSMCs were grown in 4-well chamber slides to approximately 80% confluence. Cells were stimulated for 48 hours with TGF-β1 5ng/ml and then incubated in the presence of 150 µg/ml of a PN-1 or PAI-1 blocking antibody (30 min at 37 ºC). Plasmin (10 nM) was then added and apoptosis was estimated after 18 hours using TUNEL staining. TUNEL was performed on cultured cells and aortic tissue (6 TAA, 7 AAD, 4 controls)

**Statistical analysis**

Values are expressed as means ± S.D. For results expressed as box plots, the median is shown. The n numbers are indicated for each experiment in the figure legend. Upper and lower limits of boxes represent interquartiles (75th and 25th), whereas upper and lower bars show the 95th and 5th percentiles. Since the number of experiments and point dispersion are limited, cell biology results are represented by bar graphs. The significance of differences between groups was tested using the Mann Whitney non-parametric test. A value of p≤0.05 was considered significant.

**References**

