Protease Nexin-1 Interacts With Thrombomodulin and Modulates Its Anticoagulant Effect
Marie-Christine Bouton, Laurence Venisse, Benjamin Richard, Cécile Pouzet, Véronique Arocas and Martine Jandrot-Perrus

Circ Res. 2007;100:1174-1181; originally published online March 22, 2007; doi: 10.1161/01.RES.0000265066.92923.ee

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/100/8/1174

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/03/22/01.RES.0000265066.92923.ee.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Protease Nexin-1 Interacts With Thrombomodulin and Modulates Its Anticoagulant Effect

Marie-Christine Bouton, Laurence Venisse, Benjamin Richard, Cécile Pouzet, Véronique Arocas, Martine Jandrot-Perrus

Abstract—The endothelial cell membrane glycoprotein thrombomodulin (TM) plays a critical role in the regulation of coagulation. TM is an essential cofactor in protein C activation by thrombin, and a direct inhibitor of thrombin-induced platelet activation and fibrinogen clotting. Protease nexin-1 (PN-1) is a serpin synthesized and secreted by a variety of cells including endothelial cells. PN-1 bound to the cell surface through interactions with glycosaminoglycans, is an efficient inhibitor of thrombin and controls thrombin-induced cell responses. An investigation of the interaction of PN-1 with TM using purified proteins and cultured human aortic endothelial cells was performed. Purified PN-1 was observed to bind to purified TM in a concentration-dependent manner. Double immunofluorescence studies indicated that PN-1 and TM were colocalized at the endothelial cell surface from which they were coprecipitated. Pretreatment of the cells with chondroitinase ABC greatly decreased the amount of the PN-1 associated to TM at the cell surface demonstrating the involvement of the TM chondroitin-sulfate chain in the formation of complexes. The inhibitory activity of the PN-1/TM complexes on the catalytic activity of thrombin, and on thrombin-induced fibrinogen clotting, was markedly enhanced when compared with the inhibitory activity of each partner. PN-1-overexpressing human aortic endothelial cells and PN-1-underexpressing human aortic endothelial cells exhibited respectively a significantly reduced ability and enhanced capacity to activate protein C. Furthermore, PN-1 decreased the cofactor activity of TM on thrombin activable fibrinolysis inhibitor activation by thrombin. These data show for the first time that PN-1 forms complexes with TM and modulates its anticoagulant activity. (Circ Res. 2007;100:1174-1181.)

Key Words: protease nexin-1 ■ thrombin ■ thrombomodulin ■ serpins ■ endothelial cells

Thrombin is the only protease in the coagulation cascade that possesses both coagulant and anticoagulant activities. The anticoagulant action of thrombin is dependent on thrombomodulin (TM), a transmembrane glycoprotein predominantly synthesized by vascular endothelial cells. The extracellular domain of TM consists of an N-terminal lectin-like domain followed by 6 EGF-like domains and a serine/threonine-rich region which contains potential sites for O-linked glycosylation supporting the attachment of a chondroitin-sulfate (CS) moiety. Thrombin/TM complex formation prevents thrombin-induced fibrinogen clotting, activation of Factor V and of platelets.1 In contrast, TM-bound thrombin activates protein C (PC) to generate the anticoagulant active protein C (APC).2 The TM/thrombin complex can also activate the latent inhibitor of fibrinolysis, Thrombin Activable Fibrinolyis Inhibitor (TAFI).3 Furthermore, TM exhibiting an attached CS moiety binds thrombin ~10 times tighter and accelerates inactivation of bound thrombin by antithrombin4,5 and PC inhibitor.6 The wide distribution of TM within the vascular system,7 and the marked changes in the activity pattern of thrombin on complex formation with TM, explain the major role of this protein, in the physiological anticoagulant mechanism of control in the hemostatic system.

The catalytic activity of thrombin can be inhibited by a variety of serine protease inhibitors (serpins), including antithrombin (AT), heparin cofactor II (HCII), the plasminogen activator inhibitor-1 (PAI-1) and protease-nexin-1 (PN-1). PN-1, a 43 to 50 kDa glycoprotein, is a potent inhibitor of thrombin and also inhibits other serine proteases such as, u-PA (urokinase-plasminogen activator), t-PA (tissue-type-plasminogen activator) and plasmin. However, in the presence of glycosaminoglycans (GAGs), such as heparin, thrombin becomes the preferential target of PN-1.8 In the presence or absence of heparin, PN-1 is a more potent thrombin inhibitor than AT.9 In contrast to AT and HC II, PN-1 is barely detectable in plasma.10 PN-1 is synthesized and secreted by a variety of cells including vascular smooth muscle cells,11 endothelial cells,12 human foreskin fibroblasts,13 human skeletal muscle myotubes14 and glial cells or neurons.15

Original received September 14, 2006; resubmission received February 21, 2007; revised resubmission received March 14, 2007; accepted March 14, 2007.
From the INSERM, U698, CHU Xavier Bichat (M.-C.B., L.V., B.R., V.A., M.J.-P.), Paris, France; IFR 02, Faculté Médecine Xavier Bichat (C.P.), Paris, France.
Correspondence to Dr Marie-Christine Bouton, Unité INSERM U698, CHU Xavier Bichat, 46 rue Henri Huchard 75877 Paris Cedex 18, France. E-mail mcbouton@bichat.inserm.fr
© 2007 American Heart Association, Inc.
Circulation Research is available at http://circres.ahajournals.org
DOI: 10.1161/01.RES.0000265066.92923.ee

Downloaded from http://circres.ahajournals.org/ by guest on March 1, 2014
At the cell surface, PN-1 forms SDS-stable equimolecular complexes with target proteases. Once formed, these complexes are rapidly internalized and degraded as has been reported in human foreskin fibroblasts. PN-1, therefore, has been suggested to be an important specific regulator of protease activities in the pericellular environment.

The aim of this study was to determine whether PN-1 could interact with TM, thereby improving the efficiency of thrombin inactivation by endothelial cells. For this purpose, an investigation of the capacity of TM and PN-1 to interact under purified conditions as well as on endothelial cells was performed. Furthermore the effects of TM/PN-1 complexes on the regulation of thrombin activity were analyzed by using wild-type endothelial cells or endothelial cells that both overexpress or underexpress PN-1.

Materials and Methods

Cell culture, immunofluorescence, cell transfections, enzymatic treatment of cells, reverse transcription and quantitative real-time polymerase chain reaction, TAFI activation, statistical analysis, antibodies and reagents are described in the online data supplement, available at http://circres.ahajournals.org.

Binding of PN-1 to Thrombomodulin

Binding of PN-1 to rabbit TM was analyzed in 96-multiwell plates (Immuno L, Dynatech, Chantilly, Va); Rabbit TM (0.5 μg/well in 50 mmol/L bicarbonate buffer, pH 9.6) was allowed to adsorb for 18 hour at 4°C. After saturation with 1% BSA in phosphate-buffered saline (PBS), pH 7.5, recombinant PN-1 (1.25 to 100 μmol/L in PBS, 0.1% Tween-20, 0.1% BSA) was incubated with immobilized TM for 90 minutes at room temperature, in the absence or presence of various competitors: heparin, fucoidans, polybrene, heparin- or chondroitin-sulfates. After washing, bound PN-1 was detected using a polyclonal rabbit anti-PN-1 antibody (10 μg/ml) followed by peroxidase-coupled secondary anti-rabbit IgG and OPD. The absorbance at 492 nm was monitored in a microtiter plate reader (iEMS, Labsystem, Courtaboeuf, France). Values for Kd were calculated as described previously.

The uncatalyzed second order rate constant, ie, in the absence of thrombomodulin, was determined using the above method but with PN-1 (150 μmol/L) present in the medium.

Thrombin Inhibition at the Surface of Endothelial Cells

Thrombin (0.5 μmol/L in TBS-20 mmol/L Tris, 150 mmol/L NaCl pH 7.5- containing 2.5 μmol/L CaCl2, and 0.1% PEG 8000) was incubated with confluent cells which have been or not pre-incubated with PN-1 (5 or 10 nmol/L) and rinsed before use. In other experiments, thrombin was incubated with chondroitinase-treated confluent HAEC monolayers. After 10 minutes incubation of thrombin on confluent cells, aliquots were removed and transferred into a microtiter plates containing S-2238 (0.3 mmol/L). Thrombin activity was measured as above and residual thrombin activity was calculated.

Fibrinogen Clotting

Fibrinogen (2.5 mg/ml in 10 mmol/L Imidazole, 150 mmol/L NaCl, 10 mmol/L CaCl2, 0.1% PEG 8000, pH 7.5) was mixed with different concentrations of thrombomodulin and PN-1. After 5 minutes at 37°C, clotting was initiated by the addition of 1 nmol/L thrombin. The time to clot formation was measured using a KC 10 automatic coagulometer. A standard curve (α-thrombin 0.125 mmol/L to 2 mmol/L) was used to calculate the percentage of residual thrombin activity.

Protein C Activation in a Fluid Phase Assay

Thrombin (0.5 μmol/L) was incubated for 10 minutes at 37°C with rabbit thrombomodulin (0.5 μmol/L) in TBS containing 10 mmol/L CaCl2, and 0.1% PEG 8000, in absence or presence of PN-1 (0.25 to 2 nmol/L). Bovine protein C (80 mmol/L) was added and the incubation continued at 37°C. At specified timed, aliquots were removed; thrombin was inactivated by 100 U/ml hirudin, and activated protein C (APC) was measured using 0.2 mmol/L S-2366 in TBS, pH 7.5.

Protein C Activation at the Endothelial Cell Surface

Thrombin (0.5 mmol/L in TBS containing 2.5 mmol/L CaCl2 and 0.1% human serum albumin) was incubated for 10 minutes with confluent cells which have been or not preincubated with PN-1 (5 or 10 nmol/L) and rinsed before further use. In other experiments, thrombin was incubated with PN-1- over or underexpressing cells or with control cells. Protein C (80 mmol/L) was, then added, and the incubation at 37°C was continued for 90 minutes. Activated protein C was quantified as described above.

Results

PN-1 Binds to TM

A concentration-dependent and saturable binding of PN-1 to immobilized rabbit TM was observed with an apparent Kd of 105.1±4.9 mmol/L (mean±SD) (Figure 1A). Heparin (20 μg/mL) inhibited PN-1 binding to TM by 83.4%±7.3% (Figure 1B).

High-molecular weight fucoidans (20 μg/mL) are sul-
3.2% (Figure 1B). Finally, chondroitin-sulfate/H11006 by 71.8% glycan-dependent interactions, blocked PN-1-TM interaction 6.8%. TM being 111.7%/H11006 on PN-1 binding to TM, the percentage of PN-1 binding to the capacity to bind to vitronectin19,20 and to inhibit thrombin serpin synthesized by endothelial cells that shares with PN-1 the binding of PN-1 to TM (Figure 1B). PAI-1 is another

calized with PN-1 on the surface of HAECs (Figure 2A).

Double immunofluorescence studies revealed that TM colo-
and anti-TM antibodies and confocal microscopy analysis.

Cells was analyzed by immunofluorescence using anti-PN-1

PN-1 and Thrombomodulin Are Complexed at the Surface of Endothelial Cells

The localization of PN-1 and TM at the surface of endothelial cells was analyzed by immunofluorescence using anti-PN-1 and anti-TM antibodies and confocal microscopy analysis. Double immunofluorescence studies revealed that TM colocalized with PN-1 on the surface of HAECs (Figure 2A).

When cells were pre-treated with chondroitinase ABC, we observed a robust decrease in the labeling with the anti-PN-1 antibody whereas the labeling with the anti-TM antibody remained unchanged (Figure 2B) leading to a disruption of the protein colocalization. This indicates that the CS chain on TM is important for its colocalization with PN-1.

To determine whether PN-1 and TM colocalization was caused by protein association, immuno-precipitation experiments were performed using either the anti-PN-1 or anti-TM monoclonal antibodies. A band at ~50 kDa, corresponding to PN-1 was detected in the samples precipitated by the anti-TM antibody (Figure 3A) and reciprocally a spread signal at ~70 kDa corresponding to TM was present in the sample precipitated by the anti-PN-1 antibody (Figure 3C) confirming that the two proteins were complexed. Pretreatment of HAEC with chondroitinase ABC resulted in a decreased intensity of the PN-1 band in the cell lysates (Figure 3B) and in the TM immuno-precipitate (Figure 3A). After the treatment with chondroitinase, TM was still present in cells but detected as a narrow band, because of the loss of the highest molecular weight species of TM after deglycosylation (Figure 3D).

Inhibition of Thrombin Activity by the TM-PN-1 Complexes

Because CS appeared to be involved in PN-1 binding to TM and are known to accelerate thrombin inhibition by PN-1,21 the effect of TM on the inactivation of thrombin by PN-1 was investigated in a fluid phase assay. The uncatalyzed rate constant for thrombin inhibition by PN-1 was $6.6 \times 10^7 \pm 0.2 \times 10^5$ M$^{-1}$s$^{-1}$, and is in agreement with previous
thrombin inhibition by PN-1. Polybrene (50 μg/mL) accelerated approximately 20-fold thrombin concentration (Figure 4A). At an equimolecular ratio of S2238 hydrolysis by thrombin decreased as a function of incubated with HAECs pretreated with 5 nmol/L or 10 nmol/L PN-1, the clotting time was further increased to 329 sec in the presence of 1 nmol/L PN-1. In the presence of increasing amounts of the TM/PN-1 mixture. At 2 nmol/L PN-1, the thrombin residual activity decreased respectively to 78.8%±2.8% and 73.6%±0.4% (Figure 5A), indicating that the additional PN-1 bound to HAEC surface increased the inhibitory capacity of the cells. Interestingly, when thrombin was incubated with chondroitinase ABC-pretreated HAECs, the catalytic activity of thrombin was not blocked anymore, the residual activity being of 97.8%±4.7% (Figure 5B), indicating that the chondroitinase treatment abolished the inhibitory effect of cells on thrombin.

Inhibition of Protein C Activation by TM-PN-1 Complexes

As TM is a critical cofactor for thrombin-mediated activation of PC, an investigation of the effect of PN-1 on APC production was performed in a fluid phase assay (Figure 6A).
PC was efficiently activated by an equimolar (0.5 nmol/L) mixture of thrombin and TM, whereas in the absence of TM, thrombin failed to generate APC. PN-1 decreased the rate of protein C activation by TM/thrombin. The rate of PC activation was reduced by 2-fold in the presence of PN-1 (0.5 nmol/L), APC generation being of 6.1 ± 1.2 pmole/min in the presence of PN-1 versus 13.1 ± 1.2 pmole/min in the absence of PN-1.

Inhibition of Protein C Activation at the Surface of Endothelial Cells

PC activation was also performed on HAEC. In such conditions, HAECs were the source of thrombomodulin. The rate of protein C activation was reduced by 34.7% ± 4.7% on HAECs which have been preincubated with 10 nmol/L PN-1 (Figure 6B). To better address the functional contribution of PN-1/TM interaction on the cell surface, PN-1 siRNA was used to transiently knock-down PN-1 expression. In another set of experiments, a vector containing the complete PN-1 coding sequence was used to overexpress PN-1 (Figure 7A). We observed that 48 hours after transfection, PN-1-underexpressing HAECs exhibited a significantly enhanced capacity to activate protein C (30% ± 18% increase) and reciprocally, PN-1-overexpressing HAECs exhibited a significantly reduced ability to activate protein C (24% ± 4% decrease) (Figure 7B).

Inhibition of Thrombin Activatable Fibrinolysis Inhibitor Activation

In addition to protein C activation, the thrombin/TM complex mediates TAFI activation, a procarboxypeptidase U that

PC activation was also performed on HAEC. In such conditions, HAECs were the source of thrombomodulin. The rate of protein C activation was reduced by 34.7% ± 4.7% on HAECs which have been preincubated with 10 nmol/L PN-1 (Figure 6B). To better address the functional contribution of PN-1/TM interaction on the cell surface, PN-1 siRNA was used to transiently knock-down PN-1 expression. In another set of experiments, a vector containing the complete PN-1 coding sequence was used to overexpress PN-1 (Figure 7A). We observed that 48 hours after transfection, PN-1-underexpressing HAECs exhibited a significantly enhanced capacity to activate protein C (30% ± 18% increase) and reciprocally, PN-1-overexpressing HAECs exhibited a significantly reduced ability to activate protein C (24% ± 4% decrease) (Figure 7B).

Inhibition of Thrombin Activatable Fibrinolysis Inhibitor Activation

In addition to protein C activation, the thrombin/TM complex mediates TAFI activation, a procarboxypeptidase U that

PC activation was also performed on HAEC. In such conditions, HAECs were the source of thrombomodulin. The rate of protein C activation was reduced by 34.7% ± 4.7% on HAECs which have been preincubated with 10 nmol/L PN-1 (Figure 6B). To better address the functional contribution of PN-1/TM interaction on the cell surface, PN-1 siRNA was used to transiently knock-down PN-1 expression. In another set of experiments, a vector containing the complete PN-1 coding sequence was used to overexpress PN-1 (Figure 7A). We observed that 48 hours after transfection, PN-1-underexpressing HAECs exhibited a significantly enhanced capacity to activate protein C (30% ± 18% increase) and reciprocally, PN-1-overexpressing HAECs exhibited a significantly reduced ability to activate protein C (24% ± 4% decrease) (Figure 7B).
relatively poor activator of TAFI. In the present study, TAFI activation was completely blocked in the presence of 10 nmol/L PN-1. On 10 minutes activation of TAFI, a mixture of thrombin and TM, in the absence or the presence of TM and polybrene reversed the effect of TM.

Two other serpins, AT and the protein C inhibitor, have already been shown to bind to different sites on TM. TM has been shown to enhance the rate of thrombin inactivation by AT. AT has been shown to have a higher affinity for CS than AT. PN-1 binds to TM with a higher efficacy than AT does. PN-1 protects the fibrin clot against lysis. Thrombin alone is a relatively poor activator of TAFI. In the present study, TAFI activation was performed by an equimolar (10 nmol/L) complex of thrombin/TM for 0, 10 or 60 minutes at 37°C in the absence (–PN-1) or presence of 10 nmol/L PN-1 (+PN-1). The reaction was stopped by the addition of 2% SDS and the samples analyzed by SDS-PAGE followed by silver staining. The migration of TAFI (56 kDa), activated TAFI (36 kDa) and products of degradation of TAFI (25 kDa and 11 kDa) is indicated.

Figure 8. PN-1 inhibits TAFI activation by thrombin-thrombomodulin. TAFI (450 nmol/L) was activated by an equimolecular (10 nmol/L) complex of thrombin/TM for 0, 10 or 60 minutes at 37°C in the absence (–PN-1) or presence of 10 nmol/L PN-1 (+PN-1). The reaction was stopped by the addition of 2% SDS and the samples analyzed by SDS-PAGE followed by silver staining. The migration of TAFI (56 kDa), activated TAFI (36 kDa) and products of degradation of TAFI (25 kDa and 11 kDa) is indicated.

Discussion
The results of the present study demonstrate that PN-1 binds to TM and that the CS moiety of TM is critical for the interaction. These proposals are supported by the following evidences: First, PN-1 bound to immobilized TM. Second, PN-1 binding to thrombomodulin was inhibited by various polysaccharides such as CS and by the polycation, polybrene. Third, thrombin inhibition by PN-1 was accelerated in the presence of TM and polybrene reversed the effect of TM.
coprecipitation. In contrast, no difference in the colocalization or coprecipitation was observed after heparinase treatment (data not shown). Therefore, the efficacy of PN-1 binding to TM may be related to the TM content in CS which is variable depending on the vascular origin.30 Interestingly, in the present report, HAECs have been shown to limit the catalytic activity of thrombin by both a CS- and PN-1-dependent mechanism. Thus, TM and PN-1 variations are likely to determine the anti-thrombin activity of the endothelium. Therefore, the amount of TM/PN-1 complexes at the endothelial cell surface may vary according to the vascular territory. Whether or not PN-1 expression is variable in different vascular beds remains to be established.

When TM and PN-1 at concentrations too low to prolong the thrombin clotting time were mixed, a synergistic effect on clot formation was observed as indicated by a sharp prolongation of the clotting time. This striking anticoagulant activity of the TM/PN-1 complex is explained by the observation that TM prevents fibrinogen binding to the thrombin exosite 1,31 and enhances the inhibition by PN-1 of fibrinogen proteolysis by thrombin. The TM/PN-1 complex thus inhibits clotting in a fashion similar tohirudin, with the simultaneous blockade of the thrombin exosite 1 and catalytic site.

On another hand, TM increases the rate of PC activation by thrombin by a factor \(\approx 1000\). A recent study demonstrated that the affinity of PC for the thrombin/TM complex is determined in a primary way by active site dependent interactions.32 In the present report, PN-1 was shown to reduce the rate of PC activation by thrombin in the presence of thrombomodulin, not only in a fluid phase assay but also at the surface of endothelial cells. Indeed, transient knock-down or overexpression of PN-1 respectively increased or reduced PC activation. Membrane bound–PN-1 has thus a direct influence on PC activation and therefore a significant impact on the antithrombotic properties of endothelial cells.

TM is also a cofactor for the thrombin-catalyzed activation of TAFI. The active form of TAFI prevents fibrinolysis by removing lysine residues from fibrin. The present study indicates that PN-1 blocks TAFI activation by thrombin/TM and thus may favor fibrinolysis. Consequently, PN-1 can modulate the regulation of both the coagulation and fibrinolytic cascades by its interaction with TM. Nevertheless, the net effect of the PN-1/TM interaction on the different activities of thrombin remains to be determined.

In summary, PN-1 binds to endothelial cell TM. This interaction appears to account for an important improvement in the reactivity of the serpin with thrombin. The coordinated action of TM with PN-1 has direct consequences on thrombin activity among which a dramatic enhancement in the inhibition of fibrinolysis. The net effect of thrombin on endothelial cells might thus be regulated by the concentration of the TM/PN-1 complexes. Because TM and PN-1 are both expressed in the vasculature, local variations of expression of PN-1 and/or TM, are likely determinants for the regulation of thrombin activity in vivo.

Acknowledgments

We acknowledge Dr Ann Gils for providing the recombinant TAFI, Dr Daniel Hantai for the polyclonal antibody anti-PN-1, and Pr Denis Monard for the recombinant rat PN-1. We thank Dr Charles Brink for critical reading of the manuscript.

Sources of Funding

This work was supported by grants from INSERM, Université Paris 7 and Fondation de France n° 2006005672. Benjamin Richard was supported by training grants from the Groupe d’Etude sur l’Hémostase et la Thrombose (GEHT) and the Société française d’Hématologie (SFH).

Disclosures

None

References

ONLINE DATA SUPPLEMENT

Materials

Human α-thrombin was purified as previously described 1. Recombinant thrombin activable fibrinolysis inhibitor, TAFI-AT (Ala^{147}-Thr^{325}) 2 was a generous gift from Dr A. Gils (Laboratory for Pharmaceutical Biology and Phytopharmacology, Leuven, Belgium). Recombinant rat PN-1 (a generous gift from Dr D. Monard, Friedrich Miescher Institute, Basel, Switzerland) was produced in yeast as previously described 3. The specific siRNA duplexes targeted against human PN-1 were purchased from Ambion (Austin, TX, USA). The monoclonal antibody (1F6) directed against human PN-1 sequence was obtained by immunizing mice with the cDNA encoding human PN-1 in collaboration with Agrobio (La Ferté St Aubin, France). Anti-human PN-1 IgGs were selected for their ability to bind PN-1 in ELISA assays. IgGs were purified from ascites by chromatography on a HiTrap-protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). The polyclonal rabbit anti-PN-1 antibody was a gift from Dr D. Hantaï (Paris, France). Bovine protein C was from Enzyme Research Laboratories (South Bend, IN, USA). Rabbit TM and the goat anti-rabbit TM IgG were from American Diagnostica (Greenwich, CT, USA). The monoclonal anti-human TM IgG was from Abcam (Cambridge, UK). Alexa 568-conjugated goat anti-mouse IgG, Alexa 488-conjugated rabbit anti-goat IgG and Oligofectamine were from Invitrogen (Cergy Pontoise, France). Heparin was from Sanofi-Aventis (Paris, France). Bovine serum albumin (BSA), polybrene (Hexadimethrine bromide), a synthetic quaternary polyamine used to neutralized glycosaminoglycans, high molecular weight fucoidans, human plasma fibrinogen, heparan- and chondroitin-sulfate, protease inhibitor cocktail for mammalian tissues, chondroitinase ABC, O-Phenylenediamine Dihydrochloride (OPD) were from Sigma-Aldrich (Saint Quentin-Fallavier, France). Hirudin was from Serbio (France). The chromogenic
Thrombomodulin and PN-1 are complexed on HAEC substrates S-2238 (H-D-Phe-pipecolyl-Arg-p-nitroanilide) and S-2366 (pyro-Glu-Pro-Arg-p-nitroanilide) were purchased from Biogenic (Mauguio, France). Protein A/G-coated magnetic beads were from Ademtech (Pessac, France), horseradish peroxydase coupled secondary antibodies from Jackson ImmunoResearch (West Grove, PA, USA) and ECL from Amersham Bioscience (Uppsala, Sweden).

Methods

Cell culture.

HAECs (human aortic endothelial cells, pooled donors) were purchased from Cambrex (Rockland, ME, USA) and cultured according to manufacturer’s procedures. All experiments were carried out by the 2nd to 5th cell passage.

Enzymatic treatment of cells.

Chondroitinase ABC was used to specifically cleave chondroitin-sulfate from the endothelial cell surface. The enzyme was used at a concentration of 0.2 U/mL for 45 min in the cell incubator. The efficacy of TM deglycosylation was assessed by western blot using a lectin (Bandeiraea simplicifolia) that binds specifically to the N-acetyl-D-galactosamine residue present on chondroitin-sulfate.

Immunocytochemical analysis.

Human aortic endothelial cells (HAECs) were seeded on gelatine-coated glass coverslips and enzymatically treated or not as described above. Cells fixation was performed in 2 % paraformaldehyde at room temperature for 10 minutes followed by PBS containing 5 % BSA for 1 h. After washings in PBS, cells were incubated overnight at 4°C with the anti-human PN-1 monoclonal antibody and the anti-rabbit TM polyclonal antibody (20 µg/mL and 50 µg/mL respectively in PBS containing 0.5 % BSA). After washings in PBS, cells were incubated with Alexa 568-conjugated goat anti-mouse IgG and Alexa 488-conjugated rabbit
anti-goat IgG for 2 h at room temperature, mounted and visualized with a confocal laser-scanning microscope (LSM-510-META, Zeiss, Mannheim, Germany) equipped with a x63 oil-immersion objective. Simultaneous two-channel recording was performed with a pinhole size of 1.00 Airy Units by using excitation wavelengths of 488 and 588 nm. The specificity of the labelling was proved by the absence of signal when the primary antibody was omitted or when using an irrelevant antibody. The colocalization of the two antibodies was analyzed using the co-localization Zeiss LSM 510 3.2 Image Browser software.

**Expression plasmids of PN-1 and transfection.**

The cDNA coding sequence for human PN-1 preceded by a Kozak consensus translation initiation site was inserted as a KpnI/EcoRV fragment into a pcDNA3 expression vector (Invitrogen, Cergy Pontoise, France). The pcDNA3 vector containing the PN-1 coding sequence or an empty pcDNA3 vector (mock) were transfected into HAECs by using FuGENE 6 (Roche Applied Science, Meylan, France) according to the manufacturer’s instructions. Cells treated with the transfection reagents alone are referred to as “control cells”. Cells were assayed after 48 hours of transfection.

**siRNA and cell transfection.**

The following pre-designed annealed siRNA was chosen for PN-1 silencing: sense sequence 5’-GGUUUUUCAUCAAGUGUGt-3’ and antisense sequence 5’-CACAAUCUGAUGAAACCt-3’. The pre-designed annealed irrelevant siRNA from Eurogentec (Searing, Belgium) was used as negative control. The duplexes (150 pmol per well of 12-well cell culture plates) were introduced into subconfluent (~ 80-85 %) cultured HAECs using Oligofectamine reagents according to the manufacturer’s instructions. Cells
treated with the transfection reagents alone are referred to as “control cells”. Cells were assayed after 48 hours of transfection. To verify PN-1 extinction, RNA were collected 48 h post-transfection and analyzed by quantitative RT-PCR.

**Reverse transcription and quantitative real-time polymerase chain reaction.**

Total RNA was extracted with Trizol (Invitrogen, Cergy Pontoise, France) and was reverse-transcribed using the Superscript II Reverse transcriptase (Invitrogen, Cergy Pontoise, France) as previously described. The resulting cDNA was used as a template for quantitative PCR analysis of PN-1 and GAPDH mRNA expression in a LightCycler system with SYBR Green detection (Roche Applied Science, Mannheim, Germany). PN-1 primers were: forward 5'-CCGCTGAAAGTTCTTGG-3' and reverse 5'-CAGCACCTGTAGGATTATGTGCG-3'. The following run protocol for PN-1 was used: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 60°C, 10 sec; 72°C, 20 sec. The lightCycler run protocol for GAPDH was as follows: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 65°C, 10 sec; 72°C, 20 sec. GAPDH primers were: forward 5'-GGGCACCCTGGGCTAAACTGA-3' and reverse 5'-TGCTCTTGCTGGGGCTGGT-3'. The level of mRNA encoding PN-1 was normalized relative to GAPDH mRNA level.

**Activation of TAFI.**

Recombinant TAFI (450 nmol/L) was incubated with 10 nmol/L thrombin and 10 nmol/L thrombomodulin in 30 µl TBS containing 5 mmol/L CaCl₂ and 0.1 % Tween 80 at 37°C for 0, 10, or 60 min, in the presence or absence of 10 nmol/L PN-1. The reactions were stopped at the indicated time points by the addition of 2% SDS and used for SDS-PAGE followed by silver staining.

**Statistical analysis.**
Results are shown as means ± SD. Statistical evaluation was performed using a student’s t-test. *P* values < 0.05 were considered statistically significant.

**References**


