Macrophages and Platelets Are the Major Source of Protease Nexin-1 in Human Atherosclerotic Plaque

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Objective—Protease nexin-1 (PN-1), a serpin constitutively expressed by vascular smooth muscle cells and endothelial cells, inhibits thrombin, plasminogen activators, and plasmin and can thus be expected to play a role in vascular biology. The present study addressed the question of PN-1 expression in human atherothrombosis.

Methods and Results—Immunohistochemistry and biochemical studies confirmed that PN-1 was expressed at a moderate level in the medial layer of normal human arteries and showed that PN-1 expression was increased in atherothrombotic lesions. In early noncomplicated plaques, PN-1 was associated with infiltrating mononuclear cells. A strong PN-1 signal was observed in advanced lesions, principally in intraplaque hemorrhage-related structures. Monocytes/macrophages and platelets were identified as the main sources of PN-1 within atherothrombotic material. Isolated human monocytes and platelets both expressed high levels of active PN-1, and monocyte PN-1 expression was upregulated, at both messenger and protein levels, in response to stimulation by lipopolysaccharides. In contrast, PN-1 expression was downregulated during their differentiation into macrophages which were shown to produce degraded forms of PN-1.

Conclusions—Platelets and monocytes/macrophages are a major source of PN-1 in human atherothrombotic plaques. PN-1 could thus represent a new actor in the evolution of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2008;28:1844-1850)

Key Words: protease nexin-1 ■ atherosclerosis ■ inflammatory cells ■ thrombin

A disequilibrium between proteases and antiproteases increasingly appears to be critical in the pathogenicity of atherothrombosis, via the modulation of the size of the plaque, its stability, and its progression toward rupture. Serine protease inhibitors, termed serpins, may be important in regulating protease activity in arterial lesions. Although plasma antithrombin could theoretically diffuse into the inner vessel layer when endothelial permeability is increased, no thrombin–antithrombin complexes could be detected in the vessel wall.1 There is substantial evidence that plasminogen activator inhibitor (PAI)-1, an essential serpin of the fibrinolytic system, may contribute to the development of coronary artery disease.2 However it is difficult to draw global conclusions regarding the role (protective or deleterious) of PAI-1 in the development of atherosclerosis.

Vascular endothelial cells3,4 and smooth muscle cells5 produce another serpin named protease nexin-1 (PN-1). PN-1 is a powerful inhibitor of thrombin and also inhibits urokinase-type plasminogen activator (uPA), tissue plasminogen activator (tPA), and plasmin. Its action is potentiated by glycosaminoglycans (GAGs) such as heparan sulfates.6 In contrast to antithrombin and PAI-1, PN-1 is barely detectable in plasma but is produced by various cell types. It was first identified in the central nervous system as glia-derived nexin and has been shown to inhibit the effects of thrombin on neuronal, as well as glial cells.7 Pericellular serpins play a pivotal role in the physiology of tissue serine proteases. Catabolism of PN-1/protease complexes occurs in fibroblasts by internalization and intracellular degradation.8 Recently PN-1 has been shown to inhibit Factor VII–activating protease (FSAP), which is known to inhibit neointima formation and vascular smooth-muscle cell proliferation by cleavage of platelet-derived growth factor-BB (PDGF-BB).9 Previous studies from our laboratory have demonstrated that PN-1 is expressed by vascular cells in a tightly regulated manner, inhibits adhesion and migration of vascular smooth-muscle cells,10,11 and amplifies thrombomodulin function on endothelial cells.4 Moreover, PN-1 has been previously shown to be associated with smooth muscle cells, macrophages, and platelets in advanced carotid atherosclerotic lesions.12 Taken together, these data support the hypothesis that PN-1 could have a regulatory role in the development of vascular lesions.

We investigated the localization and expression of PN-1 in normal human arteries compared to human atherosclerotic...
carotid arteries (noncomplicated and complicated lesions, with or without intraplaque hemorrhage) by immunohistochemical and biochemical approaches. The role of blood cells, and specifically that of platelets and monocytes as sources of PN-1, was further characterized in vitro. Freshly isolated peripheral blood platelets, monocytes, and cultured monocyte-derived macrophages were analyzed for their content in PN-1 transcript and protein.

**Materials and Methods**

**Materials**

Twenty-two human carotid endarterectomy samples and 4 nonatherosclerotic endarteries (internal mammary arteries and aorta from patients undergoing cardiac surgery) were obtained from the Centre Cardiologique Nord (Saint-Denis, France) and Hôpital Bichat (Paris, France). These tissue samples are considered as surgical waste in accordance with French ethical laws (L.1211 to 3 to L.1211 to 9) and were collected with the authorization of the French Biomedicine Agency.

Reagents, antibodies, biochemical analysis of atherosclerotic lesions, blood cell isolation and culture, LDL oxidation and uptake by macrophages, reverse transcription and quantitative real-time polymerase chain reaction (PCR), flow cytometry, Western blot, and statistical analysis are described in the supplemental materials (available online at http://atvb.ahajournals.org).

**Carotid Sampling and Preparation**

One piece of each carotid sample was treated for paraffin embedding. Immunohistochemistry is described in the supplemental materials. The culprit part of carotid samples (usually the termination of the common carotid artery) was dissected into fibrous cap, necrotic core, and residual media, as previously described by our group.13

**Results**

**PN-1 Is Expressed by Smooth Muscle Cells in the Medial Layer of Normal Human Arteries**

The pattern of PN-1 expression in normal human arteries was analyzed by immunohistochemistry. PN-1 was homogeneously expressed in the medial layer, essentially associated with smooth muscle cells (SMCs) (Figure 1B through 1D). A similar distribution of PN-1 was observed in human mammary arteries and aortae (Figure 1E and IF). Controls for immunostaining using isotype-matched IgG were consistently negative (Figure 1A).

**Characterization of Atherosclerotic Lesions**

Atherosclerotic lesions were easily characterized macroscopically and classified by histological examination after staining with hematoxylin and eosin or with Masson trichrome. The different stages of plaque evolution were determined in accordance with the classification established by Stary et al and features including the presence of a fibrous cap, evidence of inflammation, intraplaque hemorrhage, neovascularization, and lipid content were identified. There was evidence for collagen and elastic fiber fragmentation by Masson stain.

**PN-1 Expression Is Increased in Human Atherosclerotic Plaques: Similarities and Differences Between PN-1 and PAI-1 Expression**

Immunohistochemical analysis indicated that the intensity of the PN-1 signal was markedly increased in atherothrombotic carotids as compared to control arteries. In noncomplicated plaques (type III lesion) a particularly strong PN-1 signal was associated with the early stages of atheroma development (Figure 2A and detailed legend supplemental Figure II). In more advanced lesions (type V), a strong PN-1 signal was observed in the core of the lesion (Figure 2B and detailed legend supplemental Figure II), in particular in the interface between the core and the shoulder. In some plaques, a thrombus was observed within which the PN-1 signal was predominant (Figure 2C and detailed legend supplemental Figure II). Tissues showed no staining with the respective control IgG (not shown). Numerous previous studies have reported that PAI-1 was present in the atherosclerotic plaque. Because PAI-1 and PN-1 target some common enzymatic activities, we compared the expression patterns of these two inhibitors. Immunohistochemistry demonstrated that PN-1 and PAI-1 localized in the same areas, in particular within the core and the fibrotic cap (Figure 2A through 2F and detailed legend supplemental Figure II).

Immunoblot analysis of tissue extracts derived from atherosclerotic plaques revealed a major band of 50 kDa corresponding to PN-1 (Figure 3A and detailed legend supplemental Figure III) which was barely detectable in nonatherosclerotic arteries (Figure 3A and detailed legend supplemental Figure III). PN-1 was also detected by immu-
nablot in both conditioned media (Figure 3B and detailed legend supplemental Figure III) and tissue extracts (Figure 3C and detailed legend supplemental Figure III) derived from the compartments (fibrous cap, core, and remaining medial layer) obtained from several advanced plaques. PN-1 level was higher in fractions derived from the core than in fractions derived from the medial layer or the cap, in agreement with the immuno-histochemical results. In addition to a major band migrating at 50 kDa corresponding to native PN-1, bands of lower molecular mass (30 to 25 kDa) were detected and may correspond to degradation products. Moreover, the presence of high molecular weight bands in nonreducing conditions indicated that PN-1 formed complexes with other molecules within the plaques (Figure 3C and detailed legend supplemental Figure III), which differ from covalent complexes made by serpins and their target proteases because they were dissociated after reduction of the disulfide bridges (Figure 3C and detailed legend supplemental Figure III). PAI-1 was also detected by immunoblot in conditioned media, as a major band migrating at 50 kDa, and a 30kDa band corresponding to a degradation product (Figure 3D and detailed legend supplemental Figure III).
PN-1 Is Colocalized With Platelets in Advanced Atherosclerotic Plaques

Immunohistochemical analysis for PN-1 and the platelet marker CD41 was performed in both early and advanced atherosclerotic plaques. Whereas early lesions were negative for CD41 (Figure 4-1C and 4-1D and detailed legend supplemental Figure IV), some areas within advanced plaques were clearly positive for CD41 indicating the presence of platelet-derived material (Figure 4-1G and 4-1H and detailed legend supplemental Figure IV). PN-1 immunostaining was clearly associated with these CD41 positive areas (Figure 4-1-E and 4-1F and detailed legend supplemental Figure IV).

The presence of GPIIb, a marker of platelets, was assessed by immunoblot in the different compartments (cap, core, and media layer) of the atherosclerotic plaque. A 120-kDa band corresponding to GPIIb was predominantly detected in the extracts from core compartments (Figure 4-2 and detailed legend supplemental Figure IV). In addition, the core of the lesion was found to be positive for fibrin staining, and for thrombin/prothrombin and PN-1 immunostaining (Figure 4-3I through 4-3K and detailed legend supplemental Figure IV).

PN-1 Is Colocalized With Macrophages in Atherosclerotic Plaques

Immunohistochemical analysis revealed that PN-1 staining was also associated with cells positive for the macrophage-specific marker CD68, both in early and in complicated atherosclerotic lesions (Figure 5). In early plaques, CD68-positive areas were observed indicating the presence of some infiltrated macrophages in the vessel wall (Figure 5C through 5D). Moreover, some macrophages exhibiting an important phagocytic activity, as revealed by Pearl Prussian blue staining (not shown), were observed and were positive for PN-1 labeling (Figure 5G and 5J).

PN-1 Is Expressed in Isolated Platelets

The results reported above suggested that platelets could represent a source of PN-1 in atherosclerotic lesions. Therefore PN-1 expression was analyzed by flow cytometry, immunoblot, and real-time PCR in washed blood platelets. The presence of PN-1 transcripts in platelet mRNA was observed by RT-PCR (not shown). A significant shift to the right of the platelet fluorescence was observed by flow cytometry with the anti–PN-1 antibody when compared to an irrelevant isotype-matched antibody, indicating that PN-1 was present at the platelet surface. (Figure 6A and detailed
supplemental legend Figure VI). Moreover, PN-1 was detected by immunoblot analysis, in quiescent platelet lysates, as a major ≈50 kDa band (Figure 6B and detailed legend supplemental Figure VI). Interestingly, immunoblot analysis of the lysates and supernatants from thrombin-activated platelets revealed that PN-1 was secreted after platelet activation (Figure 6B and detailed legend supplemental Figure VI).

**PN-1 Is Expressed in Blood Monocytes and Monocyte-Derived Macrophages**

The results reported above also suggested that monocytes/macrophages could be a source of PN-1 within atherothrombotic lesions. However, monocytes have not been shown to express PN-1. We thus investigated PN-1 expression in highly purified monocytes isolated from human blood. The absence of contaminating platelets was indicated by the fact that CD41 was undetectable in isolated monocytes by flow cytometry, RT-PCR, and Western blot. In flow cytometry, a significant shift to the right of the fluorescence of the monocytes was observed with anti–PN-1 when compared to an irrelevant isotype-matched antibody, indicating that PN-1 was present at the monocyte surface. (Figure 6C and detailed legend supplemental Figure VI). Moreover, a major band migrating at 50 kDa corresponding to native PN-1 was observed by Western blot, in the monocyte lysates (Figure 6D and detailed legend supplemental Figure VI). In addition, PN-1 transcripts were detected by RT-PCR in monocytes (Figure 6E and detailed legend supplemental Figure VI).

To check whether PN-1 present on monocytes was active, thrombin was incubated with monocytes and its residual activity was measured. We observed a ≈30% decrease in thrombin catalytic activity after incubation with monocytes. This inhibitory effect of monocytes was prevented by a blocking anti–PN-1 antibody in the presence of which thrombin residual activity reached ≈91%. These data indicate that inhibition of thrombin by monocytes is mediated by active PN-1 expressed at their surface.

In atherothrombotic lesions, monocytes are submitted to stimulation by the proinflammatory environment and differentiate into macrophages and foam cells. We have thus investigated the regulation of PN-1 expression in activated monocytes and in macrophages. Stimulation of human monocytes with 1 μg/mL lipopolysaccharide (LPS) for 4 hours resulted in a significant increase in the intensity of the bands corresponding to native PN-1 and higher molecular mass complexes, as detected by immunoblot (Figure 6D and detailed legend supplemental Figure VI). Quantification of PN-1 transcripts was performed relative to a calibration curve established with known amounts of PN-1 cDNA. The PN-1 mRNA level was also increased (≈20-fold) in the LPS-treated monocytes as compared to control monocytes (Figure 6E and detailed legend supplemental Figure VI).

When monocytes were allowed to differentiate into macrophages, the PN-1mRNA level was decreased by ≈24-fold in macrophages compared to monocytes (Figure 6E and detailed legend supplemental Figure VI). A downregulation of PN-1 expression was also observed at the protein level on immunoblots as the bands corresponding to native PN-1 and to high molecular mass species were considerably reduced, and bands of lower molecular mass at ≈40 kDa and ≈30 kDa...
were observed (Figure 6D and detailed legend supplemental Figure VI). However, in flow cytometry analysis, macrophages displayed at their surface a similar mean fluorescence intensity (MFI) for PN-1 to that of monocytes.

In the presence of oxidized LDL (oxLDL), a fraction of the macrophages showed intracellular lipid deposits, whereas others remained unchanged (not shown), as previously described by Klinkner et al.\textsuperscript{15} Treatment of macrophages with oxLDL induced a 4-fold increase in PN-1 mRNA (Figure 6E and detailed legend supplemental Figure VI). Flow cytometry analysis revealed 2 populations: 1 with a MFI similar to that of untreated macrophages and 1 with a high MFI, indicating that 1 subpopulation of treated macrophages expressed high levels of PN-1 on their surface (Figure 6C and detailed legend supplemental Figure VI). Immunoblot analysis revealed that treatment of macrophages by oxLDL partially restored PN-1 expression. The intensity of the 50-kDa band corresponding to native PN-1 was increased as was the intensity of the bands of lower molecular mass (Figure 6D and detailed legend supplemental Figure VI).

Discussion

The contribution of PAI-1 to atherosclerosis, atherothrombosis, and neointimal growth has been largely studied but remains ambiguous. Apart from differences in genetic backgrounds, this could be attributable to the fact that numerous serine proteases present in the lesions may vary both as a function of the stage of plaque evolution and with respect to the presence of additional serpins. Our observational study suggests that in addition to PAI-1, PN-1 could represent another relevant physiological actor in the regulation of serine protease activities within vascular lesions. Both PAI-1 and PN-1 inhibit plasminogen activators, plasmin and thrombin. To date, PAI-1 is the most effective inhibitor of plasminogen activators known, with second-order inhibition rate constants of approximately $10^7$ mol/L$^{-1}$s$^{-1}$,\textsuperscript{16} whereas PN-1 is the most effective inhibitor of thrombin activity, with second-order inhibition rate constants of approximately $10^8$ mol/L$^{-1}$s$^{-1}$.\textsuperscript{17} Thus these 2 serpins are probably the main tissue regulators of plasmin generation and thrombin activity, respectively, and so play complementary roles.

Our data obtained in normal human arteries confirmed that PN-1 is physiologically expressed by vascular smooth muscle cells in vivo. Immunohistochemical studies of atherosclerotic plaques indicate that the presence of PN-1 within lesions globally increases with the stage of plaque evolution, the highest PN-1 level being detected in the core of the lesion. The presence of PN-1 in the advanced lesions and its distribution within the different compartments is confirmed by immunoblot analysis. Whatever the compartment, a significant proportion of PN-1 is present in the form of high molecular mass complexes which are dissociated after reduction of the disulfide bridges. Such complexes are also observed in the case of PAI-1 (not shown) and could correspond to aggregates formed on protein oxidation within the lesions. Serpins contain free cysteines and are well known to form aggregates; they can also associate with various proteins.\textsuperscript{18} Indeed, thiol isomerases, like platelet protein disulfide isomerase (PDI), are known to catalyze the formation of serpin-protease complexes\textsuperscript{19} by catalyzing disulfide oxidation, reduction, and isomerization. Nevertheless, we cannot rule out the presence of stable complexes between PN-1 and proteases which are not efficiently detected with the anti–PN-1 antibody used in the Western blots.

In complicated plaques, PN-1 colocalized with areas positive for the platelet antigen GPIIb/IIIa, thrombin/prothrombin immunoreactivity, and fibrin staining. This strongly suggests that these areas correspond to advanced thrombi. On the other hand, these areas presented signs of intraplaque hemorrhages (data not shown) in agreement with previous observations.\textsuperscript{13} The association of PN-1 with platelet-rich regions in the atherosclerotic plaques is in agreement with the data of Kanse et al.\textsuperscript{12} PN-1 expression by platelets has already been suggested by indirect approaches using the detection of complexes formed with radiolabeled thrombin.\textsuperscript{20} Here, we demonstrate that PN-1 is expressed by isolated peripheral platelets using immunologic approaches and the detection of the PN-1 transcript. Because of its high affinity for thrombin, platelet PN-1 could play a role in regulating thrombin activity in these areas of the plaque, enriched in components of blood origin.

The main source of PN-1 in the early stages of plaque evolution appears to be monocytes/macrophages, as indicated by the colocalization of PN-1 and CD68 labeling. Because there was no direct evidence that monocytes express PN-1, we have focused our attention on this question. Here we show for the first time that human monocytes express PN-1 at both the transcript and protein levels. At least a fraction of PN-1 is bound to the monocyte surface. LPS is an inflammatory endotoxin known to modulate the expression of numerous genes in monocytes, such as tissue inhibitors of metalloproteinases (TIMPs)\textsuperscript{21} and inflammatory genes like cytokines and chemokines.\textsuperscript{22} The stimulation of monocytes with LPS consistently increased the transcription and synthesis of PN-1 as indicated by real-time PCR and immunoblotting. Monocyte differentiation into macrophages resulted in a downregulation of PN-1 expression at both transcript and protein levels. However, membrane exposure of PN-1 appears to be similar on monocytes and macrophages, as indicated by flow cytometry, suggesting an intracellular redistribution. Treatment of macrophages with oxLDL led to the differentiation of a subpopulation of macrophages into foam cells. PN-1 was slightly increased at both transcript and protein levels after treatment with oxLDL, but results correspond to the mean value of both control macrophages and foam macrophages. Flow cytometry evidenced one subpopulation which was highly positive for PN-1. Together these data suggest that PN-1 is upregulated in foam cells compared to unstimulated macrophages. Interestingly, bands with a lower molecular mass than PN-1 were observed in the lysates of macrophages, indicating that PN-1 might be proteolytically processed in these cells.

Macrophages are a heterogeneous cell population. Previous studies referred to polarized macrophages as M1 and M2 cells.\textsuperscript{23,24} We analyzed PN-1 expression in M1 macrophages classically activated by IFN-γ in concert with LPS, and in M2 macrophages alternatively activated by interleukin (IL)-4 and IL-13. PN-1 expression levels in proatherogenic M1 macrophages are similar to those in the antiinflammatory M2 macrophages (data not shown).

Given the complex pattern of PN-1 expression demonstrated by the complementary analysis of human atherosclerotic plaques and isolated cells, it is difficult to speculate on the role of PN-1...
in terms of plaque progression and stability. However, increased expression of PN-1 could represent a mechanism of cell defense against aggression by proteases present in the atherosclerotic lesions.13,10 Here we show in monocytes and macrophages that membrane-exposed PN-1 is upregulated by stimulation with LPS and oxLDL, respectively. In a previous study, we have shown that PN-1 in smooth muscle cells was increased in a model of hypertension.5 Taken together, our data suggest that under proatherogenic conditions such as inflammation, the presence of oxLDL, or hypertension, the different cell types present in atherosclerotic plaques respond by increasing their level of PN-1.

The study of PN-1–deficient mice25 may be helpful to clarify the role of PN-1 in atherosclerosis but with the same limitations as those encountered in the study of PAI-1–deficient mice. However, the potential in vivo role of PN-1 in mechanisms associated with extensive remodeling is suggested by the characteristic pattern of PN-1 expression in pathological events such as carcinomas,26 systemic sclerosis,27 aneurysms (personal data) and by the characteristic pattern of PN-1 expression in physiological events such as ovarian follicular development,28 and hair growth control.29 The diversity of PN-1 substrates may explain, at least in part, PN-1 involvement in these pleiotropic processes, one common denominator of which is the remodelling of the extracellular matrix.

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Disclosures

None.

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Materials

Recombinant human PN-1 was a generous gift from Dr Hantai (Paris, France). Human α thrombin was purified as previously described\(^1\). The monoclonal antibody directed against human PN-1 was obtained as previously described\(^2\). It was coupled to FITC by mixing the IgG in 50mmol/L NaHCO\(_3\), 150mmol/L NaCl (pH 9.5) with a 150-fold molar excess of FITC (Sigma) at 4°C overnight. FITC-coupled anti-PN-1 IgG was separated from free FITC by chromatography on a PD10 column (Amersham) in PBS. Isotype-matched irrelevant IgG coupled to FITC (fluorescein isothiocyanate), to PE (phycoerythrin), to PC5 (phycoerythrin cyanine 5), anti-human CD41 coupled to FITC or PC5, anti-human CD14 coupled to PE, anti-human CD64 coupled to PE, anti-human CD45 coupled to PC5 were purchased from Beckman Coulter. RPMI-1640, FCS (foetal calf serum), HSA (human serum albumin) were from Sigma. Mouse monoclonal antibodies (mAb) to the human macrophage-specific marker CD68, to the lymphocyte marker CD3, and to human α-actin, as well as non-immune mouse immunoglobulins, and the Kit “En Vision Dual Link System-HRP (DAB+)” were purchased from DAKO. Mouse monoclonal antibodies to the human platelet marker CD41 (GPIIb-IIIa) and to the neutrophil-marker CD66b, were from Immunotech. The rabbit polyclonal antibody to human PAI-1 was from Santa Cruz. The sheep polyclonal antibody to human thrombin / prothrombin was from Enzyme Research Laboratories.

Immunohistochemistry

Atherosclerotic tissue sections were obtained from 22 carotid arteries (4 corresponding to non-complicated plaques type III-IV lesion, and 18 corresponding to more advanced lesions, type V-VI). Carotid sections were fixed in paraformaldehyde and incubated with anti-CD68 (PG-M1, 1.8 µg/mL), anti-CD66b (80H3, 4 µg/mL), anti-CD41 or GPIIb-IIIa (P2, 1.7 µg/mL), anti-CD3 (UCHT1, 3 µg/mL), anti-PN-1 (10 µg/mL), anti-α-actin (1A4, 1.4 µg/mL),
anti-PAI-1 (0.5 µg/mL) or anti-thrombin / prothrombin (5 µg/mL). Immunostaining was visualized using secondary antibodies conjugated with peroxidase (HRP)-labeled polymer (Envision + Dual Link System-HRP, DakoCytomation). For negative controls, the primary antibody was replaced by an isotype-matched irrelevant IgG at the appropriate concentration. Phosphotungtisic acid hematoxylin staining (PTAH) was used for fibrin detection.  

Dissection of advanced plaques into cap, core, and media layer- Collection of conditioned media  

Tissues from eight atherosclerotic carotid plaques were dissected into intima/fibrous cap (“Cap”), core (“Co”), external media (“M”) under microscope as described previously. Briefly, tissue samples were collected and processed within 2h after surgery. The core was separated from the fibrous cap and media by curetting, using a small spatula. The medial layer was peeled from the outer surface of the specimen. The fibrous cap was identified as an intimal region of white glistening connective tissue.  

For the collection of conditioned medium, small pieces of tissue (about 2 mm³) were weighed and incubated in RPMI culture medium without serum for 24 h at 37°C (5% CO₂). For standardization, the volume of medium was adjusted to sample weight (6 mL per gram) Sample weights varied from 150 to 300 mg. Conditioned media were then collected and centrifuged and stored at -80°C until further analysis.  

Biochemical Analysis of human atherosclerotic lesions  
Tissues were next homogenized (0.5 mg tissue/ mL) in lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 3mmol/L EDTA, 6 mmol/L N-ethyl-maleimide,1% Triton X-100) containing a complete cocktail of protease inhibitors (Sigma). The lysates were clarified by centrifugation (13000 g, 10 min at 4°C) and the protein concentration from each fraction was determined (BCA protein assay, Pierce). Proteins (15 µg) were separated by SDS-polyacrylamide gels after normalization to tissue wet weight, under reducing or non-reducing
conditions and transferred to polyvinylidene difluoride (PVDF) membranes. After saturation with 5% low fat milk in PBS /0.1% Tween-20 for 1h, the membranes were incubated overnight at 4°C with monoclonal antibodies against human PN-1 (5µg/mL), GAPDH (1.6µg/mL), and platelet GPIIb (PMI-1; 10 µg/mL supplied by Dr. D. Pidard) or with a rabbit anti-human PAI-1 (0.5 µg/mL), in PBS /0.1%Tween-20. The secondary antibody was a goat anti-mouse IgG or a goat anti-rabbit IgG conjugated with peroxidase (Amersham Biosciences) revealed by chemiluminescence (ECL, Pierce).

**Blood cell isolation and culture**

*Preparation of washed platelets*

Blood from healthy adult volunteers was collected on ACD-A (38 mmol/L citric acid, 60 mmol/L sodium citrate, 136 mmol/L glucose). Washed platelets were isolated as previously described. In brief, platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 120g for 15min at 20°C. PRP was decanted and acidified to pH 6.5 with ACD-A (1/10 of volume PRP). Apyrase (25µg/mL) and prostaglandin E1 (100 nmol/L) were added to PRP before platelet sedimentation at 1200g for 15 min. The platelets were resuspended in washing buffer pH 6.5 (103 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 5 mmol/L glucose, 36 mmol/L citric acid), containing 25µg/mL apyrase and 100 nmol/L prostaglandin E1. Washed platelets were resuspended in Tyrode-Hepes buffer (5 mmol/L Hapes, 12 mmol/L NaHCO$_3$, 137 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L CaCl$_2$, 0.3 mmol/L NaH$_2$PO$_4$, 1 mmol/L MgCl$_2$, 5.5 mmol/L glucose). The platelets did not exhibit any monocyte contamination as indicated by flow cytometry using anti-CD14 antibodies.

*Human monocyte isolation and stimulation*

Blood from healthy adult donors was collected into ACD-A and mononuclear cells were isolated by Ficoll density gradient centrifugation at 400g for 30 min. Following the centrifugation, the layer of mononuclear cells was centrifuged 5 min at 400g. The cells were
then resuspended in PBS containing 2 mmol/L EDTA and centrifuged again 5 min at 200g, in
order to eliminate residual platelets. Monocytes were further purified using anti-CD14 coated
beads according the manufacturer instructions (Myltenyi Biotec.). The CD14+ cells did not
exhibit any platelet contamination as indicated by flow cytometry using anti-CD41 antibodies.
Platelet contamination was also tested by RT-PCR for GPIIb in monocyte mRNA and was
below 1%. Pure monocytes (>98%) were resuspended in RPMI medium. Monocytes (2x10^6)
were stimulated with 1µg/mL lipopolysaccharide (LPS) for 4h at 37°C in RPMI medium
containing 5% FCS.

*Human monocyte-derived macrophages*

After isolation, monocytes were resuspended in Macrophage serum-free medium
(Invitrogen) with antibiotics. 2x10^6 cells/well were plated in six-well plates and allowed to
adhere for 2h at 37°C. Non-adherent cells were then removed by washing in PBS. Adherent
cells were cultured with human granulocyte macrophage colony stimulating factor (GM-
CSF), 70U/mL (Promocell). The medium was discarded after 3 days and cells were washed
once with PBS. Macrophages were then allowed to differentiate for another 3 days in the
same conditions, but without GM-CSF.

*Human low density lipoprotein (LDL) and oxidation of LDL.*

Human LDLs (d 1.019-1.063 g/mL) were a generous gift from Dr Negre Salvayre
(Toulouse, France). After extensive dialysis against PBS for 24h, oxidation of LDL was
performed by incubating 1mg LDL/mL in PBS containing 5 µmol/L CuSO4 and by exposure
to an UV source (Osram UV-C tube, λ 254 nm , 0.5mW/cm^2) for 12h. The level of LDL
oxidation was evaluated by monitoring the formation of thiobarbituric acid-reactive
substances according to the method of Yagi and the relative electrophoretic mobility was
assessed by using Hydragel (Sébia). Under the standard conditions used here, mildly oxidized
oxLDL contained 7 nmol TBARS/ mg apoB.
**LDL	extsubscript{ox} uptake by human monocyte-derived macrophages**

Six days after monocyte plating, cells were incubated with macrophage medium supplemented with 50µg protein LDL	extsubscript{ox} per ml for 48h. Macrophages and foam macrophages were cultured, for light microscopy, on poly-D-lysine-coated glass coverslips. Cultures were fixed with formol-calcium for 10 min and then briefly immersed in 60% isopropanol. Cells were stained with 0.5% Oil red O in 70% isopropanol for 10 min and rinsed in PBS. Cell nuclei were stained by Harris hematoxylin for 15 sec rinsed in PBS and mounted for light microscopic examination. Previously established criteria were used for identifying foam cells. Foam cells were defined as macrophages in which the entire cytoplasm was filled with Oil Red O-stainable lipid droplets.

**Flow cytometry**

Platelets and monocytes were gated on Forward Scatter (FSC) and Structure Scatter (SSC) (FACS Beckman Coulter Epics XL-MCL). Cell suspensions (1x10	extsuperscript{7} cells/ml) were incubated with different primary antibodies coupled to FITC, PE or PC5 according to experimental conditions, for 30 min in the dark at room temperature. Ten thousands events were analysed for fluorescence for each sample. Mean fluorescence was compared to the mean fluorescence obtained with an irrelevant IgG.

**Reverse Transcription and real time quantitative polymerase chain reaction (RT-PCR)**

Total cell RNA was isolated from 2x10	extsuperscript{6} cells by using the Trizol reagent (Invitrogen), according to the manufacturer’s directions. Double stranded cDNAs were synthesized and amplified as previously described. Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science). Specific primers for the human PN1 gene were (sens: 5’-CCGCTGAAAGTTCTT GGCA-3’ and antisens: 5’-CAGCATTAGTGC-3’). PN-1 mRNA levels were normalized to GAPDH mRNA. Specific primers for human GAPDH were (sens: 5’-GGGCACCCTGGG CTAAACTGA-3’;
and antisens: 5’-TGCTCTTGCTGGGGCTGGT-3’). The following run protocol was used: denaturation: 95°C, 10min; amplification and quantitation (40cycles): 60°C (for PN-1) or 65°C (for GAPDH), 10sec, 72°C, 20sec. Fluorescence of the samples was monitored continuously while the temperature was increased from 60°C or 65°C to 95°C at a linear transition rate of 0.1°C s⁻¹, and arrested by a final cooling step at 45°C.

**Thrombin inhibition by monocyte-expressed PN-1**

Thrombin (0.1 nmol/L in 20 mmol/L Hepes, 150 mmoL/L NaCl pH 7.5-containing 0.1% human serum albumin) was incubated with monocytes (5x 10⁶/ well in 6-multiwell plates) which had been pre-incubated or not with a blocking-anti PN-1 antibody. After 60 minutes incubation of thrombin with monocytes, aliquots were removed and transferred into a microtiter plate containing the chromogenic substrate S-2238 (0.3 mmoL/L). Thrombin activity was determined by measuring the rate of substrate hydrolysis at 405 nm using a microtiter plate reader and the Biolyse 2 application (Labsystem).

**Statistical analysis**

Results are expressed as means ± SD. Statistical evaluation was estimated between groups by 1-way ANOVA and Mann Whitney tests. A value of P < 0.05 was considered as statistically significant.

**REFERENCES**


DETAILED FIGURE LEGENDS

**Figure II:** Comparative immunohistochemical localization of PN-1 and PAI-1 in atherosclerotic arteries. General views (X10) of the endarterectomy specimens showing PN-1 immunostaining (A-C) and PAI-1 immunostaining (D-F) were reconstituted using Cartograph software. (A and D) serial sections of a non-stenosing plaque (type III lesion), showing neointima (NI), a limited cap (Cp) and some residual medial layer (M). (B and E) serial sections of an advanced type V lesion with an important core (Co). (C and F) serial sections of a ruptured plaque, with loss of the necrotic core and an attached intraluminal thrombus (Thr).

**Figure III:** Compartmentalization of PN-1 and PAI-1 in atherosclerotic lesions.

The atherosclerotic plaques from 9 patients (P, P1-P8) were dissected into medial layer (M), cap (Cp) and core (Co). Five non-atherosclerotic carotid (C1-C5) samples were used as controls. Either tissues (A and C) or conditioned media (B and D) obtained as described in methods were analyzed by immunoblot using anti PN-1 (A-C) or anti-PAI-1(D) antibodies. Reducing conditions were applied in B, C (lower panel) and D.

**Figure IV:**

**I-** Comparative localization of PN-1 and the platelet marker CD41 in early non-complicated atherosclerotic plaques (type III lesion) (A-D) and in advanced atherosclerotic plaques (type V lesion) (E-H). PN-1 immunostaining (A,B,E,F) was performed with the monoclonal anti-human PN-1 antibody and platelet labeling (C,D,G,H) was performed with the monoclonal anti-human CD41.

**II-** Immunoblot analysis of the different compartments from 8 atherosclerotic plaque extracts (P1-P8) with an antibody against the platelet glycoprotein GPIIb. Tissue extract samples were reduced before migration in 7.5% SDS-PAGE. A platelet lysate was used as a positive control.
III- Comparative localization of PN-1, fibrin and thrombin/prothrombin. (I) General view of an advanced atherosclerotic plaque showing PN-1 immunostaining. The PN-1-positive staining within the square is shown at higher magnification. Serial sections show fibrin accumulation detected by PTAH blue staining (J) and thrombin/prothrombin staining detected using a goat anti-human thrombin/prothrombin antibody (K).

**Figure VI: PN-1 expression in human platelets, monocytes and macrophages.**

Flow cytometry of PN-1 was performed using a specific FITC-coupled monoclonal antibody on platelets (A), monocytes, and macrophages (C). Black histograms represent cells stained with isotype-matched control antibodies.

In B, lysates and supernatants of resting and thrombin activated washed platelets were analyzed by immunoblotting for PN-1 and for GPIb as platelet marker. Recombinant PN-1 (rPN-1) was used as a positive control.

In D, lysates of monocytes stimulated or not by LPS, human blood-derived macrophages, and oxLDL-treated-macrophages were prepared and subjected to immunoblot for PN-1.

In E, PN-1 mRNA was quantified by RT-PCR in monocytes, LPS-stimulated monocytes, macrophages, and oxLDL-treated-macrophages. GAPDH was used for normalization. Results are expressed as means ± SD of 3 representative independent cell preparations. (∗) p < 0.001.