Reciprocal regulation of urokinase receptor (CD87)-mediated cell adhesion by plasminogen activator inhibitor-1 and protease nexin-1

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Summary

Protease nexin-1 (PN-1) and plasminogen activator inhibitor-1 (PAI-1) are serine protease inhibitors that bind to the extracellular matrix protein vitronectin (VN) with high affinity. PAI-1 is known to inhibit cell adhesion and migration by binding to VN and inhibiting the interaction with integrins or the urokinase receptor (uPAR). Unexpectedly, PN-1 was found to increase the association between VN and uPAR in the presence of enzymatically active uPA. Through this mechanism PN-1 also stimulated uPAR-dependent cell adhesion to immobilized VN. In contrast to PAI-1, PN-1 did not influence VN binding to integrins or integrin-mediated cell adhesion. Upon adhesion of monocytes to VN there was an accumulation of uPAR and PN-1 at the interface between the cell and the matrix, whereas on fibronectin (FN) both components were distributed evenly over the whole cell as visualized by confocal microscopy. Immunohistochemistry of atherosclerotic vessels indicated that PN-1 was found associated with smooth muscle cells, macrophages and platelets. In some regions of the diseased vessels PN-1 was in close proximity to VN and uPAR, but no PN-1 was present in normal vessels. These results indicate a novel function of PN-1 linked to complex formation with uPA that leads to the regulation of VN-dependent adhesion of leukocytes.

Key words: Cell adhesion, Urokinase receptor, Vitronectin, Protease nexin-1, Serine protease inhibitors

Introduction

Pericellular proteolysis, particularly initiated by the plasminogen activator/plasmin system, fulfils pivotal regulatory functions in cellular adhesion, migration and invasion. The urokinase plasminogen activator (uPA) is a key activator of this system and its function relies on the interaction with the urokinase receptor (uPAR) (CD 87) (Behrendt et al., 1995; Blasi, 1999). Plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1) are the main inhibitors of the catalytic activity of uPA (Collen, 1999; Gloor et al., 1986). The urokinase system mediates cell surface proteolysis and chemotactic cell locomotion and concurrently regulates cell adhesion (Chapman, 1997; Ossowski and Aguirre-Ghiso, 2000). uPAR not only directly mediates leukocyte adhesion to a vitronectin (VN) substratum in the presence of uPA (Wei et al., 1994), but also regulates integrin-mediated cell adhesion (Gyetko et al., 1995; May et al., 1998; Wei et al., 1996). This suggests that the same components that are required for breaking cellular contacts with the substratum are also responsible for forming new adhesion points. The focalized assembly of these components, their relative concentrations, and their activation status determine the extent of cell adhesion.

uPAR-dependent cell adhesion to VN is regulated by a number of factors, which interact with either VN or uPA, such as PAI-1 (Deng et al., 1996; Kanse et al., 1996). PAI-1 binds to VN with high affinity (Podor et al., 2000) and competes for uPAR as well as for integrin binding to VN (Deng et al., 1996; Stefansson and Lawrence, 1996). The formation of a macromolecular complex between PAI-1 and VN stabilizes the active conformation of the serine protease inhibitor. This interaction is also the basis for PAI-1-mediated inhibition of cell adhesion and migration (Stefansson and Lawrence, 1996). PN-1, the other inhibitor of uPA, can also bind to VN either alone or together with the inactivated target protease (Rovelli et al., 1990). uPA-PN-1 complexes bind to uPAR and are internalized through the low density lipoprotein receptor-related protein (LRP) (Conese et al., 1994). PN-1 is found in platelets (Gronke et al., 1989), fibroblasts (Farrell et al., 1988) and in the central and peripheral nervous system (Mansuy et al., 1993). The expression of PN-1 is highly upregulated during situations such as nerve injury (Meier et al., 1989). Owing to its broad specificity, PN-1 inhibits uPA, plasmin, trypsin, thrombin and other serine proteases (Stone et al., 1987), and these interactions with proteases can be augmented by heparan sulphate proteoglycans (Rovelli et al., 1992). Moreover, PN-1 inhibits thrombin-stimulated cell division (Festoff et al., 1996), migration of cerebellar granular cells (Lindner et al., 1986) or uPA-dependent extracellular matrix degradation (Donovan et al., 1994) and promotes neurite outgrowth (Gloor et al., 1986). PN-1 knockout mice exhibit

defects related to male fertility (Murer et al., 2001), but the underlying molecular mechanisms for these cellular functions of PN-1 remain undefined.

Since both PAI-1 and PN-1 bind VN directly and PAI-1 exerts an anti-adhesive effect, we have examined the role of PN-1 in cell adhesion. In vitro binding experiments with purified components indicated that PN-1, in contrast to PAI-1, promotes the interaction between VN and uPAR in the presence of active uPA and the same phenomenon was recapitulated in cell adhesion assays. Immunolocalization of PN-1 and uPAR to cell adhesion patches as well as to regions of VN deposition in atherosclerosis provides a further basis for the hypothesis that the regulation of cell adhesion is a novel function of PN-1.

Materials and Methods

Proteins and antibodies

VN was isolated as previously described (Kanse et al., 1996). Recombinant PN-1 and the anti PN-1 mAb 4B3 were produced as described before (Stone et al., 1994). Recombinant soluble uPAR, and its truncated form lacking domain I, were generously provided by Dr N. Behrendt (Finsen Laboratory, Copenhagen, Denmark). Integrin $\alpha_v\beta_3$ was given by Dr S. Goodman (Merck, Darmstadt, Germany). The mAb to VN, 13H1 and VN7, were provided by Dr P. Declerk (Leuven, Belgium). Human two-chain active uPA was from Medac (Hamburg, Germany). Recombinant Gly158 ScuPA (non-cleavable inactive mutant) of high molecular mass uPA and anti-uPA mAb 4D1E8 were a kind gift from Dr H. R. Lijnen (Leuven, Belgium). The amino-terminal fragment of uPA (ATF, amino acids 1-135) was from American Diagnostica (Greenwich, CT). Recombinant active PAI-1 was from Dr J. Deinum (Astra Hässle AB Mölndal, Sweden). Heparin and bovine thrombin were from Sigma (Taufkirchen, Germany).

Cells

U937 (myelomonocytic cells) and BAF3 (interleukin-3-dependent mouse B-cells) were obtained from American Type Culture Collection (Rockville, MD) and were cultured in RPMI-1640 medium containing fetal calf serum (FCS) 10% (v/v), penicillin (100 U/ml) and streptomycin (100 μg/ml), respectively. The culture medium for BAF3 cells was supplemented with interleukin-3 (2 ng/ml). uPAR-transfected BAF3 cells were prepared as described before (Chavakis et al., 1999). U937 cells were differentiated with vitamin D3 (50 nM) and transforming growth factor β (1 ng/ml) overnight.

Binding interactions in a purified system using ELISA

Maxisorp microtiter 96-well plates (Nunc, Roskilde, Denmark) were coated at a concentration of 2-5 μg/ml each with $\alpha_V\beta_3$ integrin, VN, uPA, uPAR or truncated uPAR (lacking domain 1) in TBS and subsequently blocked with 3% (w/v) bovine serum albumin (BSA). Binding of 1-2 μg/ml of multimeric VN or uPA to the immobilized proteins was performed in the absence or presence of competitors as indicated in the figure legends. Binding to $\alpha_V\beta_3$ integrins was performed exactly as described before in the presence of divalent cations (Kanse et al., 1996). The bound VN was detected using the mAbs VN7 or 13H1, and the bound uPA was detected with mAb 4D1E8, at a concentration of 125 ng/ml each and the quantitation was achieved with peroxidase-linked secondary rabbit anti-mouse IgG. The absorbance at 405 nm was monitored in a microtiter plate reader (Molecular Devices, Menlo Park, CA). The binding of ligands to BSA-coated wells was used as a blank in all the experiments and was subtracted to obtain the receptor specific binding.

Binding interactions in a purified system in the solution phase using co-immunoprecipitation

PN-1, VN, suPAR, uPA, PAI-1 and ATF were incubated in different combinations in TBS with 1% (w/v) BSA for 2 hours at 22°C. Thereafter mAbs to VN, uPAR, PN-1 or uPA were added and further incubated for a further 2 hours. The resulting complexes were captured by adding protein A/G Sepharose (Amersham-Pharmacia, Freiburg, Germany) and the immunoprecipitates were analyzed by western blotting with a rabbit polyclonal anti-uPAR IgG.

Cell adhesion assays

Ninety-six-well cell culture plates (Nunc) were coated with 2 μg/ml multimeric VN and blocked with 3% (w/v) BSA. Differentiated U937, BAF3 or uPAR-BAF3 cells (suspension cultures) were washed and resuspended in RPMI 1640 with 0.3% (w/v) BSA. The cell suspension was added to the plates (100,000 cells/well) with test substances and incubated for 60 minutes at 37°C. The wells were washed and the number of adherent cells was quantified using Crystal Violet staining. The dye was extracted from cells with acetic acid:methanol:water (10:30:60, v/v) and its absorbance measured at 590 nm.

Enzyme activity assays

These were performed using the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-para-nitro-anilide) for thrombin and S-2444 (pyro-Glu-Gly-Arg-para-nitro-anilide) for uPA (Chromogenix, Mölndal, Sweden) using a kinetic multiplate reader.

Confocal laser scanning microscopy

PN-1 was labeled with rhodamine (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions and the conjugate was dialyzed extensively against PBS. Permanox slides (Nunc, Roskilde, Denmark) were coated with VN (2 μg/ml) or fibronectin (FN; 10 μg/ml) in PBS overnight at 4°C and thereafter blocked with 3% (w/v) BSA in PBS. Differentiated U937 cells were washed extensively in serum-free medium and were then preincubated for 1 hour at 37°C with uPA (50 nM) and/or rhodamine labeled PN-1 (3 μg/ml). After extensive washing, the cells were allowed to adhere to the VN/FN-coated slides for 1 hour at 37°C. Thereafter the slides were washed twice with PBS and fixed with ice cold methanol-acetone (1:1, v/v). After blocking with 3% (w/v) BSA the slides were incubated with anti-uPAR IgG (rabbit polyclonal, 3 μg/ml) or rabbit preimmune IgG. After washing, secondary FITC-coupled IgG (Dianova, Hamburg, Germany) was added (1:100 to 1:200). After extensive washing, the cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Confocal microscopy was performed using a spectral laser scanning microscope (Leica Microsystems, Heidelberg, Germany) using plan-Apo 63× objectives (NA 1.4 oil). After defining the top and the bottom plane of the cells, images of red and green fluorescence in approximately 50 sections each of 0.2 μm thickness were captured using software supplied by the manufacturer. Double stained images were merged to analyze the extent of colocalization of the different antigens.

Immunohistochemistry

Atherosclerostic tissue sections were obtained from five carotid arteries, collected during carotid eversion endarteriectomy. Additionally, tissues from a ruptured aortic aneurysm and four normal femoral and internal mammary arteries were investigated. Specimens were fixed with 4% (w/v) paraformaldehyde/PBS immediately after surgical excision and then paraffin embedded and cut in 6 μm serial sections on a Leica SM 2400 microtome. After mounting on poly-L-lysine-coated slides, the sections were deparaffinized in xylene and

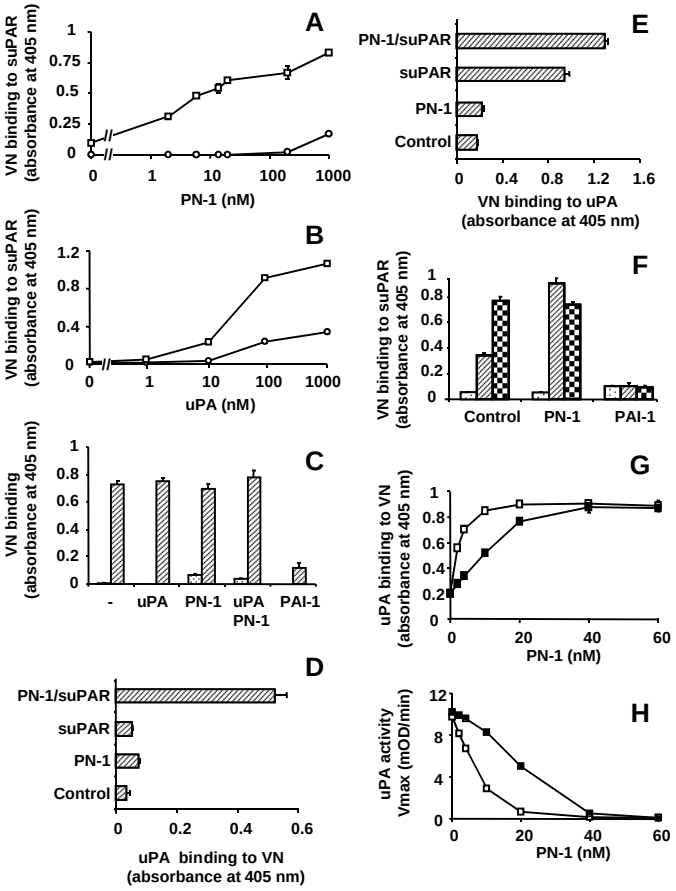

Fig. 1. Characteristics of the interactions between uPA, VN, suPAR and PN-1 using ELISA. (A) Binding of VN (1 μg/ml) to suPAR-coated wells (5 μg/ml) in the absence (open circle) or presence (open square) of uPA (20 nM) and increasing concentrations of PN-1. (B) Binding of VN to suPAR-coated wells in the absence (open circle) or presence (open square) of PN-1 (250 nM) and increasing concentrations of uPA. VN binding was detected with the mAb 13H1. (C) Binding of VN (1 μg/ml) to suPAR (D2 + D3; 5 μg/ml)-coated wells (dotted bars) or $\alpha_V\beta_3$ (5 μg/ml)-coated wells (hatched bars) in the absence or presence of uPA (100 nM), PN-1 (100 nM), PAI-1 (100 nM) or a combination of uPA and PN-1. VN binding was detected with the mAb 13H1. (D) Binding of uPA (20 nM) to VN-coated wells (5 μg/ml) was tested in the absence (control) or presence of either PN-1 (100 nM), suPAR (50 nM) or a combination of the two as indicated. uPA binding was detected with the mAb 4D1E8. (E) Binding of VN (2 μg/ml) to uPA-coated wells (5 μg/ml) was tested in the absence (control) or presence of either PN-1 (100 nM), suPAR (50 nM) or a combination of the two as indicated. VN binding was detected with the mAb VN-7. (F) Binding of VN (1 μg/ml) to suPAR-coated wells (5 μg/ml) was measured in the absence (control) or presence of PN-1 (200 nM) or PAI-1 (100 nM), without any further addition (dotted bars) or together with enzymatically active uPA (20 nM; hatched bars) or enzymatically inactive Gly158-ScuPA (20 nM; chequered bars), respectively. VN binding was detected with the mAb 13H1. (G) Binding of uPA (20 nM) to VN-coated wells (5 μg/ml) in the presence of suPAR (50 nM) and increasing concentrations of PN-1 was tested in the absence (open square) or presence of thrombin (20 nM) (solid square) as indicated. Binding was detected with the mAb 4D1E8. (H) At the end of the incubation period in the above experiment the supernatants were removed and then analyzed for proteolytic activity using the chromogenic substrate S-2444, which is specific for uPA but not for thrombin. Results from a typical experiment are shown (mean absorbance ± s.e.m. of triplicate wells), and similar data were obtained in three separate experiments. Absence of error bars indicates that the s.e.m. is smaller than the size of the symbol.

rehydrated through graded ethanol washes (100%-30%). Slides were blocked with 5% (v/v) normal goat serum and then incubated with anti-uPAR (mAb R4, 2 μg/ml) anti-VN (mAb VN7, 1 μg/ml), anti-uPA (mAb 4D1E8, 5 μg/ml), anti-PN-1 (mAb 4B3, 3 μg/ml), anti-smooth muscle α actin (1A4, Dako, 1:100), anti-CD68 (KP1, Dako, 1:100), rabbit preimmune IgG or control mAb (UPC 10) at the appropriate concentration. Ensuing incubations were carried out with biotinylated secondary antibodies, streptavidin-alkaline phosphatase-conjugate, Fast Red staining (all reagents from Sigma), and Mayer's hemalum counterstaining. Slides were covered with glycerol-gelatin and examined by light microscopy with the aid of an Olympus BH2 microscope.

Results

Influence of PN-1 on the interaction between VN and uPAR

To test the hypothesis that PN-1 influences the VN-uPAR interaction, a series of binding experiments were performed using purified components. VN bound to immobilized suPAR and this binding was enhanced in the presence of uPA. In the absence of uPA, PN-1 had a small stimulatory influence on VN binding to immobilized suPAR at concentrations higher than 300 nM. However, in the presence of a fixed concentration of uPA (20 nM), PN-1 facilitated the binding of VN to suPAR in a dose-dependent manner (Fig. 1A). Saturation of binding was observed at concentrations higher than 20 nM PN-1. Similarly, in the presence of a fixed amount of PN-1 (250 nM), the binding of VN to immobilized suPAR increased dose-dependently with uPA (Fig. 1B). Concentrations higher than 250 nM uPA lead to saturation of binding. Hence, PN-1 increased the binding of VN to suPAR by about fivefold over that by uPA alone and about an equimolar concentration of both uPA and PN-1 was required for a maximal effect.

No binding of VN or any influence of PN-1 on VN binding was observed when truncated uPAR (lacking domain I) was immobilized indicating that the effect of PN-1 was specific for intact uPAR (Fig. 1C). As expected, the other serine protease inhibitor PAI-1 was found to be a robust inhibitor of VN binding to both uPAR and $\alpha_v\beta_3$ integrin, whereas PN-1 had no influence on VN binding to $\alpha_v\beta_3$ integrin (Fig. 1C). Hence, PN-1 increased VN binding to one of its receptors, uPAR, but not to the other receptor $\alpha_v\beta_3$ integrin, indicating the specificity of this effect. Antibody-based detection was used in these experiments but similar results were obtained with ^{125}I-VN (data not shown).

To exclude any immobilization related artifacts, we tested these binding interactions in different experimental conditions i.e., instead of suPAR, VN or uPA was used as a coating. In contrast to the substantial binding of VN to immobilized suPAR, the binding of uPA to immobilized VN was low and remained so even in the presence of suPAR. However, PN-1 stimulated this binding substantially (Fig. 1D). Similarly the binding of VN to coated uPA was increased in the presence of suPAR and was further augmented by the addition of PN-1 (Fig. 1E). Depending on which of the proteins was

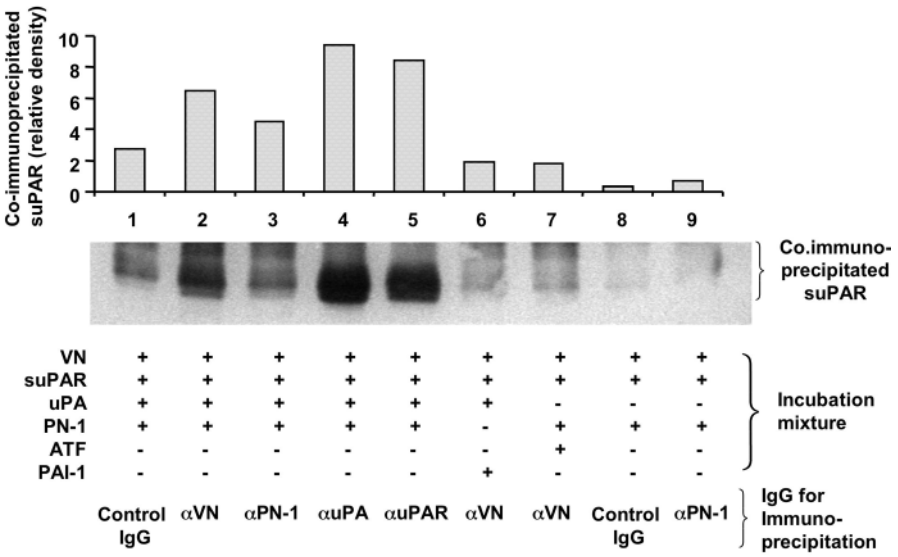

Fig. 2. Interaction between uPA, VN, suPAR and PN-1 in the solution phase. PN-1, VN, suPAR, uPA, PAI-1 and ATF (4 μg/ml each) were incubated in different combinations, as indicated, in TBS with 1% (w/v) BSA for 2 hours at 22°C. Thereafter, immunoprecipitation was performed with control mAb (lanes 1 and 8), anti-VN, 13H1 (lanes 2, 6 and 7), anti-uPAR, R4 (lane 5), anti-uPA, 4D1E8 (lane 4) or anti-PN-1, 4B3 (lanes 3 and 9) all at 4 μg/ml. The immune complexes were captured by adding protein A/G Sepharose and the immunoprecipitates were analyzed by western blotting with a rabbit polyclonal anti-uPAR followed by densitometric analysis. Similar results were obtained in three independent experiments.

immobilized, either VN or uPA or suPAR, the stimulatory effect of PN-1 was different, which might be a consequence of an immobilization-related alteration in protein conformation. Thus, PN-1 promoted the interaction between VN and uPAR in the presence of uPA under various experimental conditions (see solution phase experiments below).

To further explore how PN-1 increases VN binding to uPAR, experiments were performed in the presence of enzymatically active or inactive uPA. The enzymatically inactive form of uPA, Gly158 ScuPA was more effective in stimulating the binding of VN to suPAR than the enzymatically active uPA. PN-1 increased VN binding to suPAR only in the presence of enzymatically active uPA, but not in the presence of the inactive Gly158 ScuPA or ATF, indicating that it is the formation of a complex with active uPA that influences the VN-uPAR interaction (Fig. 1F, data with ATF not shown). Even in the presence of a range of concentrations of Gly158 ScuPA or ATF (1-100 nM), PN-1 did not influence VN binding to suPAR. The maximal increase in binding with the uPA-PN-1 complex was similar to that obtained with inactive uPA alone (Fig. 1F). In contrast, PAI-1 prevented the binding of VN to suPAR in the presence of enzymatically active, as well as non-active, uPA (Fig. 1F). The inhibition of binding in the presence of both forms of uPA indicates that the PAI-1 binding to VN, and the masking of the uPAR binding site accounts for this inhibition.

PN-1 is a potent inhibitor of thrombin, and a covalent complex between both proteins is rapidly formed. Upon inclusion of thrombin the ability of PN-1 to increase VN binding to uPAR, in the presence of uPA, was decreased (Fig. 1G). This inhibition of complex formation mirrored the neutralization of the anti-proteolytic activity of PN-1 by thrombin that rendered it unavailable for binding to uPA (Fig. 1H). In the presence of 20 nM uPA, maximum binding was achieved with 20 nM PN-1 in the absence of thrombin whereas 40 nM PN-1 was required in the presence of 20 nM thrombin. This provides indirect evidence that the optimal complex formation is achieved in the presence of equimolar concentrations of active uPA and PN-1.

These interactions were also tested in solution phase binding assays followed by co-immunoprecipitation since this procedure is free of any immobilization-related artifacts. Incubation of VN, PN-1, uPA and suPAR in the solution phase followed by immunoprecipitation with mAb to VN, PN-1 or uPA led to coimmunoprecipitation of suPAR (Fig. 2). When PN-1 was replaced by PAI-1 no coimmunoprecipitation was apparent (Fig. 2, lane 6) which correlates with the inhibitory effects of PAI-1 in the ELISA above. The replacement of enzymatically active uPA with ATF also resulted in decreased complex formation similarly to the immobilization studies above (Fig. 2, lane 7). No coimmunoprecipitation was observed when uPA was excluded from the mixture (Fig. 2, lane 9). The results of these solution phase experiments were complimentary to those obtained using immobilized proteins.

Since PAI-1 and PN-1 mediate opposite effects on the binding of VN to suPAR, we tested their combined influence on this interaction. PAI-1 could inhibit the PN-1-induced increase in VN binding to suPAR in a dose-dependent manner (Fig. 3). The PAI-1 effect could be due to a combination of two events: either, a competition with PN-1 for binding to uPA, or to binding to VN and blocking the uPAR interacting site. The latter effect is a more probable since PAI-1 also inhibits the effect of inactive uPA as seen above (Fig. 1F).

Both, PN-1 and VN have a strong affinity for heparin or heparan sulphate proteoglycans through their respective heparin binding domains. The presence of heparin slightly potentiated PN-1-induced stimulation of the VN binding to immobilized suPAR (data not shown).

Influence of PN-1 on cell adhesion

One of the main cellular consequences of the interaction between VN and uPAR is the stimulation of cell adhesion. We have developed a model system based on uPAR-transfected BAF3 cells to study uPAR-dependent adhesion phenomenon (Chavakis et al., 1999). uPAR-transfected BAF3 cells adhere strongly to VN but untransfected cells do not. PN-1 stimulated the adhesion of uPAR-transfected BAF3 cells to a VN-coated surface in the presence of uPA in a dose-dependent manner (Fig. 4A). In the presence of 50 nM uPA maximal adhesion was observed with 50 nM PN-1. There was a reduction of cell adhesion at higher concentrations of PN-1 which is in contrast

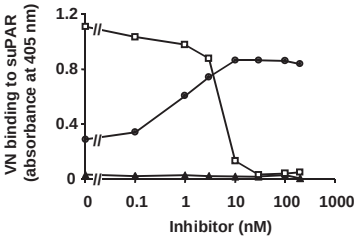

Fig. 3. Influence of PAI-1 on uPA-PN-1-induced VN binding to immobilized suPAR. Binding of VN (1 μg/ml) to suPAR-coated wells (5 μg/ml) was tested in the presence of increasing concentrations of PN-1 in the absence (solid triangle) or presence of uPA (20 nM; solid circle). For comparison, the binding of VN in the presence of uPA (20 nM) and PN-1 (250 nM) was determined in the presence of increasing concentrations of PAI-1 (open square). Binding was detected with the mAb VN-7, and data represent mean absorbance ±s.e.m. (*n*=3) of a typical experiment. Similar results were obtained in three separate experiments.

to the binding data with pure components. The β_1-integrin-dependent adhesion of these cells on FN was not influenced by PN-1 (data not shown). As in the binding assays, the stimulatory effect of PN-1 was only observed in the presence of enzymatically active uPA but not when ATF or Gly158 ScuPA were present (Fig. 4B). In contrast to the binding assays the effect of the uPA-PN-1 complex on cell adhesion was greater than that due to the inactive forms of uPA at their maximum effective concentrations. The presence of heparin (20-100 μg/ml) slightly increased the effect of PN-1-uPA complex on cell adhesion (data not shown). When PN-1 was first neutralized by complex formation with thrombin, then PN-1 could no longer influence cell adhesion in the presence of uPA (Fig. 4C). PN-1 also increased the adhesion of uPAR-expressing myelomonocytic cell line, U937, to VN (Fig. 4D) indicating that this phenomenon is ubiquitous. Taken together, the characteristics of the stimulation of cell adhesion induced by PN-1 match the in vitro binding data.

PN-1 and uPAR distribution in adherent monocytes

Since PN-1 together with uPA, promotes uPAR-dependent cell adhesion on a VN substratum we investigated the distribution of these components in adherent cells. Differentiated U937 cells adhere to VN-coated substrate through uPAR (Waltz et al., 1993) and to FN via β_1 integrins (Wei et al., 1996). The localization of exogenous PN-1 was determined by labeling the PN-1 with rhodamine, whereas uPA and uPAR were identified with anti-uPA or anti-uPAR antibody followed by a FITC-labeled secondary antibody. Differentiated U937 cells were preincubated with rhodamine-labeled PN-1 and/or uPA, extensively washed to remove unbound material, and then allowed to adhere to VN or FN. Analysis of uPAR and PN-1 by confocal microscopy showed that upon adhesion of cells to VN, uPAR was highly concentrated at the interface between the cells and the immobilized surface (Fig. 5A). We have termed this the 'adhesion patch' to distinguish it from the adhesion plaques seen in normally adherent mesenchymal or epithelial cells. On the FN-coated surface there was a uniform distribution of uPAR over the whole cell with no concentration into the interface region (Fig. 5A). The distribution of uPA

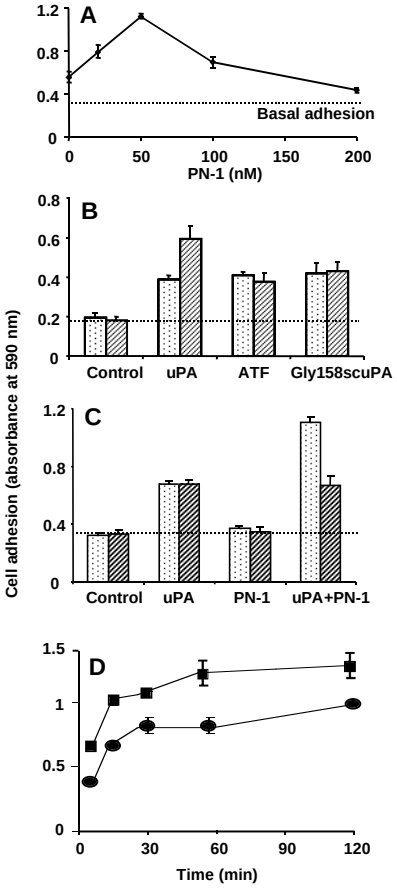

Fig. 4. Influence of PN-1 on uPAR-dependent cell adhesion on VN. (A) Adhesion of uPAR-transfected BAF3 cells to VN-coated wells (2 μg/ml) in the presence of uPA (50 nM) as well as different concentrations of PN-1. The dotted line indicates the level of basal adhesion in the absence of any test substances. (B) Adhesion of uPAR-BAF3 cells to VN-coated wells in the absence (control) or presence of uPA, ATF or Gly158ScuPA (each at 50 nM) in the absence of any further additions (dotted bars) or the presence of PN-1 (100 nM) (hatched bars). (C) Adhesion of uPAR-BAF3 cells to VN-coated wells in the absence (control) or presence of uPA (50 nM), PN-1 (50 nM) or a combination of uPA (50 nM) and PN-1 (50 nM) in the absence of any further additions (dotted bars) or the presence of thrombin (50 nM) (hatched bars). uPA and PN-1 or uPA, PN-1 and thrombin were preincubated for 60 minutes at room temperature to allow complex formation before addition to the cell adhesion assay. (D) U937 cells were differentiated overnight with vitamin D3 and TGFβ prior to the assay. Time-dependent adhesion of cells to VN in the presence of uPA (50 nM; solid circle) or uPA/PN-1 (50 nM each; solid square). Cell adhesion was measured as absorbance at 590 nm and is expressed as mean ± s.e.m. (*n*=3) of triplicate wells. Similar results were obtained in three separate experiments.

followed that of uPAR precisely on both substrata (data not shown). PN-1 was found to colocalize with uPAR and was also concentrated strongly in the adhesion patch on a VN substratum but was diffusely distributed on FN (Fig. 5A). This substratum-dependent distribution pattern of uPAR and PN-1 on VN versus FN was confirmed by quantification of fluorescence in the vertical plane (Fig. 5B). High levels of uPAR and PN-1 were also found inside the cells, especially on

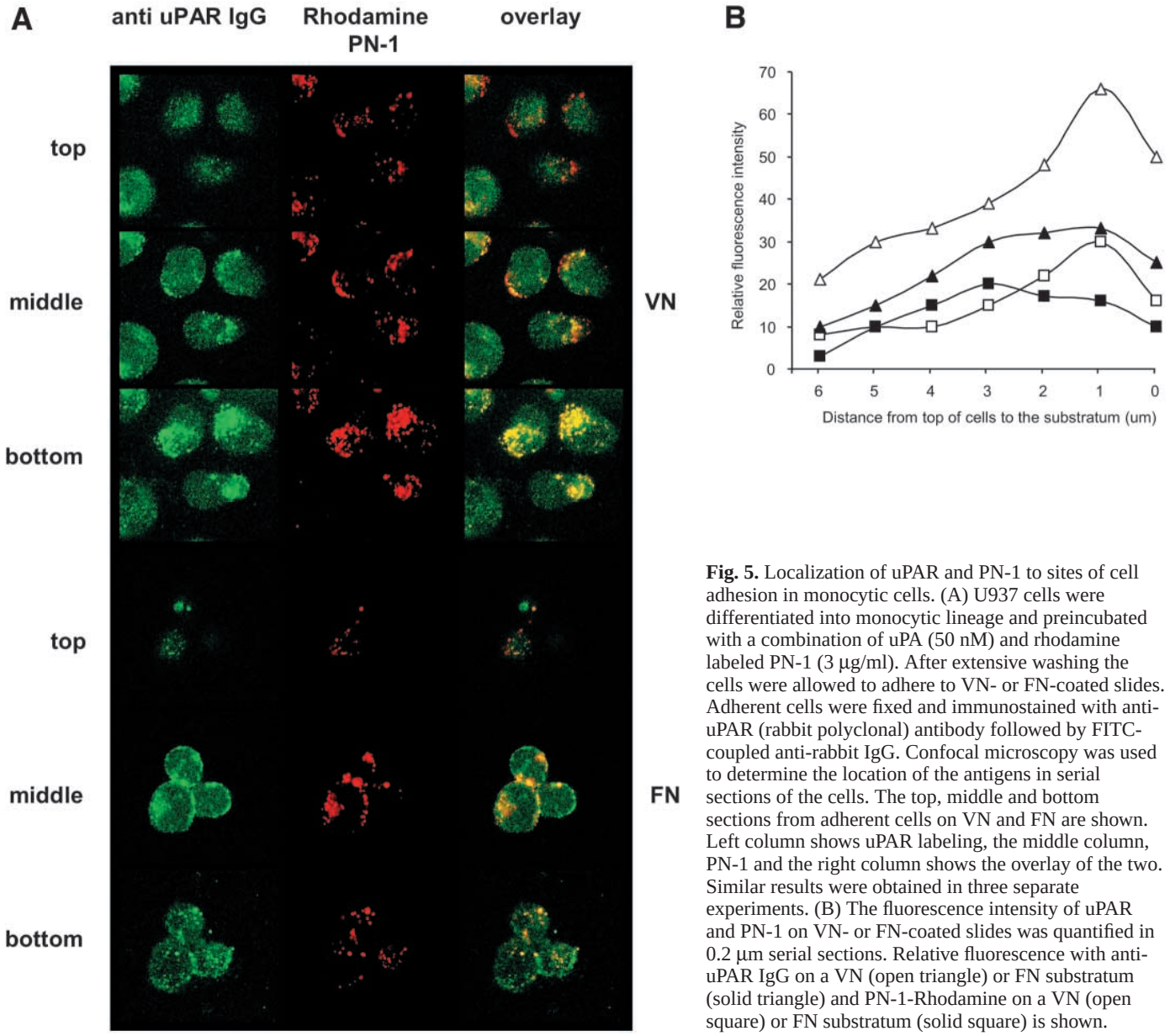

Fig. 5. Localization of uPAR and PN-1 to sites of cell adhesion in monocytic cells. (A) U937 cells were differentiated into monocytic lineage and preincubated with a combination of uPA (50 nM) and rhodamine labeled PN-1 (3 μg/ml). After extensive washing the cells were allowed to adhere to VN- or FN-coated slides. Adherent cells were fixed and immunostained with anti-uPAR (rabbit polyclonal) antibody followed by FITC-coupled anti-rabbit IgG. Confocal microscopy was used to determine the location of the antigens in serial sections of the cells. The top, middle and bottom sections from adherent cells on VN and FN are shown. Left column shows uPAR labeling, the middle column, PN-1 and the right column shows the overlay of the two. Similar results were obtained in three separate experiments. (B) The fluorescence intensity of uPAR and PN-1 on VN- or FN-coated slides was quantified in 0.2 μm serial sections. Relative fluorescence with anti-uPAR IgG on a VN (open triangle) or FN substratum (solid triangle) and PN-1-Rhodamine on a VN (open square) or FN substratum (solid square) is shown.

a FN substratum, indicating the internalization of uPAR-uPA-PN-1 complexes as reported before (Conese et al., 1994). These results indicate that PN-1 associates with uPAR, VN and uPA in adhesion patches and thus can directly play a role in regulating cell adhesion on VN.

Localization of PN-1 in atherosclerotic plaques

The above experiments indicate that PN-1 could potentially influence the adhesion of monocytic cells and recent studies indicate that it is present in the vasculature (Bouton et al., 2003). We have observed that in normal vessels low levels of uPAR and VN are found in the endothelium and the media, respectively, and there was no PN-1 immunostaining. Previous investigations have indicated that there is deposition of VN and infiltration of leukocytes in atherosclerotic plaques (Dufourcq et al., 1998). Localization of PN-1 in a series of sections from different atherosclerotic plaques showed that it was found in regions of deposited platelets. Immunostaining was also observed in regions characterized by accumulation of smooth muscle (anti-α-smooth muscle actin) and macrophage (anti-CD68) origin (Fig. 6). These were the same areas that were positively stained with antibodies to uPA, VN and uPAR. In accordance with previous findings that mesenchymal cells produce PN-1 we observed strong staining in the cytoplasm of some smooth muscle cells, the rest being associated with extracellular matrix. In the atherosclerotic plaque the presence of VN and uPAR is generally associated with active matrix turnover and cellular activation and substantial amounts of PN-1 are found at the same locations.

Discussion

Certain mesenchymal cells produce PN-1 (Farrell et al., 1988)

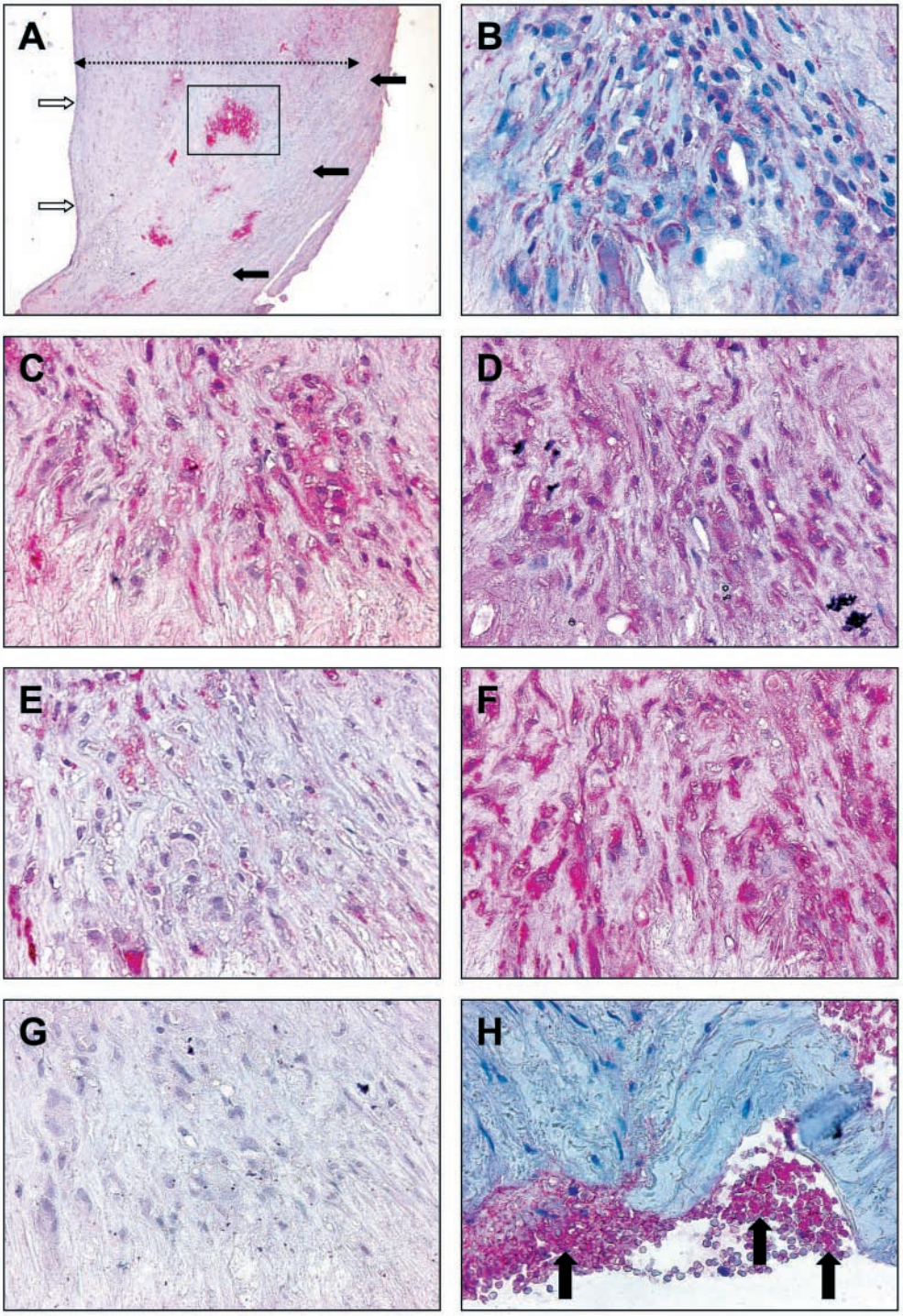

Fig. 6. Localization of PN-1 in atherosclerotic vessels. Immunohistochemistry of arterosclerotic carotid artery, visualised through streptavidin-alkaline phosphatase conjugate, Fast Red stain and Mayer's hemalum counterstain. (A) General view (×34) of the endarterectomy specimen showing VN immunostaining. The extension of the neointima is indicated by a dotted line, the luminal border is marked by white arrows, the border of the neointima with the media is marked by black arrows. (B-F) The VN-positive foam cell accumulation, marked by a rectangle, shown at higher magnification. Serial sections (×272) showing PN-1 (B), uPAR (C), uPA (D), macrophage-antigen CD68 (E) and smooth muscle α-actin (F) immunostaining. Note that PN-1 is expressed in VN-, uPAR- and uPA-positive areas (red stain). The foam cell accumulation is composed of smooth muscle cells and macrophages. Note the distribution of PN-1 that can be attributed to cytoplasmatic localization and matrix-associated protein. (G) A negative IgG control of PN-1 immunostaining of the same area (×272) was completely negative, underlining the specificity of the PN-1 immunoreactivity. (H) PN-1 was also demonstrated in a thrombus at the edge of a ruptured abdominal aortic aneurysm (×272). The dark arrows point to accumulations of PN-1-positive thrombocytes.

and platelets seem to store and secrete this protease inhibitor (Gronke et al., 1989). We found high concentrations of PN-1 in deposited platelets as well as in vascular smooth muscle cells of the vessel wall in conjunction with uPAR and VN. Similarly, the presence of PN-1 in medial smooth muscle cells of the rat aorta and its upregulation in hypertension has been described (Bouton et al., 2003). This expression or deposition of PN-1 in the vessel wall may be related to the inhibition of serine proteases or their internalization. The results presented in this study would indicate that PN-1 regulates uPAR-dependent adhesion of monocytic cells on a VN substratum. Evidence is also provided in vitro, with solid phase and solution phase binding experiments, that PN-1 increases the binding of uPAR to VN in the presence of uPA. It remains to be seen if these findings can be extended to other situations e.g., to describe the cellular effects of PN-1 on neurons (Gloor et al., 1986).

Previous studies have shown that both PN-1 and uPA bind to VN and that the affinity of the PN-1-uPA complex for VN is similar to that of the individual components (Rovelli et al., 1990). uPA has been shown to increase the affinity of binding between uPAR and VN (Deng et al., 1996; Kanse et al., 1996). Although PN-1 slightly increased the affinity of VN for uPAR by itself, the combination of uPA and PN-1 was stronger than either PN-1 or uPA alone. ATF or the inactive single chain uPA mutant were not effective in this respect indicating that enzymatically active uPA binds to PN-1, and by adopting a

different conformation it appears to be a more effective inducer of VN binding to uPAR than uncomplexed active uPA alone. The uPA-PN-1 complex stimulated binding to the same level as the enzymatically inactive uPA. Further studies are required to clarify why enzymatically inactive uPA, either alone or in combination with PN-1, is intrinsically more potent in stimulating VN-uPAR interaction than enzymatically active uPA. Since thrombin inhibited the interaction between PN-1 and uPA by binding to PN-1 this provides further evidence that PN-1 forms a complex with uPA and increases the affinity of the uPAR-VN interaction.

At lower concentrations of PN-1 the adhesion of cells to immobilized VN was stimulated, whereas at higher concentrations of PN-1 a reduction to basal levels was observed. This unusual pattern of cell adhesion was not related to the internalization of the uPA-PN-1 complex through LRP since receptor associated protein (RAP), an inhibitor of LRP, did not influence cell adhesion (data not shown). This suggests that lower levels of PN-1 promote these interactions, whereas at high PN-1 concentrations additional low affinity interactions inhibit cell adhesion. Partial evidence for this can be found in Fig. 1A where less than 100 nM PN-1, alone, did not influence VN binding to uPAR but at higher concentrations there was a significant increase in binding. Although the changes in the in vitro binding assays and the cell adhesion assays are in opposite directions they could be the result of the same events.

The adhesion of U937 cells to VN resulted in a pronounced clustering of uPAR into the areas of cell contact with the immobilized surface, leading to the formation of 'adhesion patches'. This concentration of uPAR in adhesion patches was not observed on FN where uPAR remained dispersed over the whole cell surface. The distribution of PN-1 in adherent cells mirrored that of uPAR closely, since it was also concentrated within the adhesion patches on a VN substratum but was diffusely distributed over FN. In agreement with previous reports (Conese et al., 1994), high levels of internalized PN-1 and uPAR were evident in cross sections of cells, especially those on a FN substratum. However, it appears that the PN-1 and uPAR, which are involved in cell adhesion through association with uPA and VN are protected from internalization, which might also explain that fact that the immunofluorescence associated with uPAR and PN-1 is lower on a FN substratum as compared with a VN substratum. This possibility needs more rigorous analysis. The ability of exogenous PN-1 to associate with cell surface uPAR in these adhesion patches provides additional evidence for its involvement in regulating uPAR-dependent cell adhesion. In a previous study with HT1080 cells no difference in the distribution of uPAR was observed in cells attached to a VN or FN substratum, although uPAR and β_3 integrins were co-clustered in adhesion plaques on a VN but not a FN substratum (Xue et al., 1997).

Even though PN-1 and PAI-1 bind to VN, they have opposing effects on uPAR-VN-mediated cell adhesion. This relates to the fact that PAI-1 binds largely to the amino-terminal 'somatomedin B domain' of VN (Podor et al., 2000), whereas PN-1 binds predominantly to the carboxy-terminal heparin binding domain, although this has not been fully proved (Rovelli et al., 1990). The 'somatomedin B domain' of VN is a probable binding region for uPAR as well as integrins (Deng et al., 1996). Thus, integrin-mediated activities can be inhibited by PAI-1 but not by PN-1, as demonstrated here.

In the present study we describe a novel function of PN-1, i.e. the regulation of adhesion of uPAR-expressing cells on a VN matrix. This finding is supported by the observations that (i) PN-1 facilitates the binding of uPAR to VN in the presence of uPA, (ii) PN-1, uPA and uPAR are concentrated in adhesion patches, and (iii) PN-1 enhances uPAR-mediated cell adhesion to a VN matrix. Since VN is a major constituent of the provisional matrix at sites of injury and inflammation (Gailit and Clark, 1994) and PN-1 is localized to regions of vascular remodeling in the vessel wall, the regulation of uPAR-mediated cell adhesion by the relative concentrations of PAI-1 and PN-1 is likely to be important in the adherence and recruitment of leukocytes.

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