ONLINE DATA SUPPLEMENT

Materials

Human α-thrombin was purified as previously described. Recombinant thrombin activable fibrinolysis inhibitor, TAFI-AT (Ala^{147}-Thr^{325}) 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. 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
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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’-GGUUUUCAAUCAGAUUGUGtt-3’ and antisense sequence 5’-CACAAUCUGAUUGAAACCtg-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’-CAGCACCTGTAGGATTATGTCG-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 CaCl$_2$ 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**