

Proteinase 3 Interacts with a 111-kD Membrane Molecule of Human Umbilical Vein Endothelial Cells

MIRIAM E. J. TAEKEMA-ROELVINK, CEES VAN KOOTEN, EVERT HEEMSKERK, WILLEKE SCHROEIJERS, and MOHAMED R. DAHA

Department of Nephrology, Leiden University Medical Centre, The Netherlands.

Abstract. Proteinase 3 (PR3) is the major autoantigen of anti-neutrophil cytoplasmic antibodies in Wegener's granulomatosis. Previously, it was demonstrated that PR3 induces apoptosis of human endothelial cells and that PR3 contributes to endothelial cell activation by enhancing interleukin-8 production. The present study demonstrates that PR3 binds specifically to human umbilical vein endothelial cells (HUVEC). Digoxigenin (DIG)-labeled PR3 bound readily to HUVEC cultured on coverslips. By fluorescence-activated cell sorter analysis, a homogeneous binding of PR3 to HUVEC, using either DIG-labeled or unlabeled PR3, was observed. No detectable membrane expression of PR3 was observed after either tumor necrosis

factor- α stimulation or in nonstimulated HUVEC. The binding of PR3-DIG to HUVEC was dose-dependent and was inhibited by unlabeled PR3. Scatchard analysis revealed 2000 binding sites per cell, with a K_d of 0.1 μ M. Affinity precipitation of biotin-labeled HUVEC membrane proteins with protein G-Sepharose bearing PR3 resulted in specific precipitation of a membrane molecule with a molecular weight of 111 kD under nonreducing conditions and 52 and 63 kD under reducing conditions. It is hypothesized that PR3, either released systemically or locally at inflammatory sites following activation of primed polymorphonuclear neutrophils, may lead to endothelial cell injury and activation of endothelial cells.

Wegener's granulomatosis is an autoimmune disease, characterized by crescentic glomerulonephritis and systemic necrotizing vasculitis, mainly affecting small blood vessels (1). The presence of anti-neutrophil cytoplasmic antibodies (ANCA), directed against different granule components of neutrophils, is a common feature of many systemic vasculitides. In Wegener's granulomatosis, proteinase 3 (PR3) is the main target antigen for ANCA. PR3 is a 29-kD neutral serine proteinase present in the α -granules of polymorphonuclear neutrophils (PMN). PR3 may be released from cytokine-primed PMN after activation by ANCA, as shown by *in vitro* studies (2,3). In patients with Wegener's granulomatosis, increased serum PR3 concentrations have been detected compared with healthy control subjects (4,5). Furthermore, serum PR3 levels were found to correlate with disease activity (5).

Earlier studies by Kao *et al.* (6) demonstrated that PR3 is able to induce emphysema in hamsters after intratracheal instillation, suggesting a role for PR3 in neutrophil-mediated disease. Several *in vitro* studies have provided evidence that PR3 may directly contribute to vascular injury, such as that seen in Wegener's granulomatosis. Ballieux *et al.* (7) showed that incubation of human umbilical vein endothelial cells (HUVEC) with PR3 resulted in detachment and cytolysis.

Moreover, PR3 induced apoptosis of bovine pulmonary artery endothelial cells (8), and we have recently shown that PR3 induced apoptosis of human endothelial cells (9). Berger *et al.* (10) reported that PR3 may contribute to the acute inflammatory process by enhancing interleukin-8 (IL-8) production by HUVEC. More recently it was shown that PR3, in contrast to neutrophil elastase and cathepsin G, serine proteases also present in azurophilic granules, was able to enhance the release of active tumor necrosis factor- α (TNF α) and IL-1 β from stimulated THP-1 cells, a human monocytic cell line (11).

The mechanisms involved in the interaction of PR3 with endothelial cells are not fully understood. Because PR3 enhances IL-8 production (10) and induces apoptosis (8,9), it is thought that PR3 binds to endothelial cells. In the present study, we have investigated the binding of PR3 to human endothelial cells. Our results suggest strongly that PR3 binds to a specific membrane molecule on HUVEC.

Materials and Methods

Isolation of PR3

PR3 was isolated from neutrophil azurophilic granules as described by Leid *et al.* (12). In brief, leukocytes were isolated from enriched buffy coats after hypotonic lysis of erythrocytes. Subsequently, leukocytes were resuspended in relaxation buffer (100 mM KCl, 35 mM MgCl₂, 10 mM Hepes, pH 7.3) and lysed by nitrogen cavitation. Lysed cells were collected in 25 mM ethyleneglycol-bis(β -aminoethyl ether)-*N,N'*-tetra-acetic acid, and the cell-free supernatant was obtained after centrifugation (900 \times g, 10 min). Next, α -granules were isolated from the bottom of an 84% Percoll gradient after centrifugation at 48,000 \times g for 10 min. After ultracentrifugation (174,000 \times g, 60 min), the green α -granule-containing pellet was removed from the remaining Percoll, and after centrifugation was frozen at -20°C until needed.

Received July 14, 1999. Accepted September 14, 1999.

Correspondence to Miriam E. J. Taekema-Roelvink, Department of Nephrology, Leiden University Medical Center, Building 1 C3-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: +31 (0)71 5263964; Fax: +31 (0)71 5248118; E-mail: taekema@mail.medfac.leidenuniv.nl

1046-6673/1104-0640

Journal of the American Society of Nephrology

Copyright © 2000 by the American Society of Nephrology

Azurophilic granule-containing pellets, derived from approximately 300×10^9 leukocytes, were lysed by incubation in phosphate-buffered saline (PBS) containing 1% Triton X-100. PR3 and myeloperoxidase (MPO) were isolated from the lysate by cation exchange chromatography using Biorex 70 (Bio-Rad Laboratories, Richmond, CA). The α -granule extract was dialyzed against phosphate-citrate buffer (80 mM Na_2HPO_4 , 50 mM NaCl, adjusted to pH 7.0 with 80 mM citric acid) and applied to a column of Biorex 70, which had been equilibrated and run in the same buffer. After all nonbinding proteins had washed through the column, a linear gradient up to 1 mM NaCl in starting buffer was applied. Fractions from the column were analyzed for the presence of protein (BCA protein assay; Pierce Chemical Co., Rockford, IL) and PR3, elastase, and myeloperoxidase activity, as measured by cleavage of *N*-t-BOC-L-alanine-p-nitrophenyl ester (BOC cleavage assay), S-2484, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), respectively.

PR3-containing fractions were concentrated by lyophilization and resuspended in PBS. For further purification, PR3 was applied to a Superdex 75 column (Pharmacia-Biotech, Uppsala, Sweden). Fractions were tested for the presence of protein and esterolytic activity (BOC-cleavage assay), and PR3-containing fractions were pooled, dialyzed against distilled water, and lyophilized. The lyophilized PR3 was dissolved in a minimal amount of distilled water and dialyzed against PBS. PR3 concentration was determined using a PR3 sandwich enzyme-linked immunosorbent assay (ELISA) and enzymatic activity by BOC-cleavage assay. The purity of the PR3 preparation was determined using analysis of enzymatic activity, ELISA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot. The PR3 preparation used for the experiments was not contaminated with myeloperoxidase, neutrophil elastase, or cathepsin G. SDS-PAGE analysis of the PR3 preparation revealed a triplet of bands around 29 kD.

PR3 Sandwich ELISA

A solid-phase sandwich ELISA, as described by Berger *et al.* (10), was used to measure the PR3 concentration. Briefly, 96-well microtiter plates were coated with an optimal concentration of polyclonal rabbit IgG anti-PR3 and diluted in coating buffer (74 mM NaHCO_3 , 26 mM Na_2CO_3 , pH 9.6) for 2 h at 37°C. After each incubation step, plates were washed three times with PBS/0.05% Tween 20, and all further dilutions were prepared in PBS/0.05% Tween 20/2% casein. After coating, all open binding sites on the plate were blocked using PBS/0.05% Tween 20/2% casein (30 min, 37°C). Next, twofold serial dilutions of the PR3 sample were added and tested against serial dilutions of a PR3 standard (126 $\mu\text{g}/\text{ml}$). Bound PR3 was detected using digoxigenin (DIG)-conjugated polyclonal rabbit IgG anti-PR3 in the first step, followed by horseradish peroxidase-conjugated sheep F(ab')₂ fragments of anti-DIG. Finally, the ELISA was developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), containing H_2O_2 (0.0005%), and the optical density was assessed at 415 nm. The standard curve started at a concentration of 630 ng/ml and was linear down to 5 ng/ml.

Culture of Cells

HUVEC were isolated from human umbilical cord veins according to Jaffe *et al.* (13) and cultured as described by Ballieux *et al.* (7) and Miltenburg *et al.* (14). In brief, cells were cultured on gelatin-coated tissue culture plates or flasks in M199 medium containing Earle's salts and glutamine, supplemented with 10% heat-inactivated fetal calf serum (ΔFCS), penicillin-streptomycin (100 IU/ml, 100 $\mu\text{g}/\text{ml}$) (all from Life Technologies, Paisley, United Kingdom), 0.002% en-

dothelial cell growth factor (isolated from bovine hypothalamus) (15), and 7.5 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). Cell cultures were performed at 37°C, 5% CO_2 and 95% relative humidity. Only HUVEC between passages 2 and 6 were used for experiments. Jurkat cells were cultured in Iscove's modified Dulbecco's medium with Glutamax® (Life Technologies), containing 10% ΔFCS and penicillin-streptomycin (100 U/ml, 100 $\mu\text{g}/\text{ml}$). U937 cells were cultured in RPMI supplemented with 10% ΔFCS and penicillin-streptomycin (100 U/ml, 100 $\mu\text{g}/\text{ml}$).

Detection of Binding of PR3 to HUVEC

Immunofluorescence Staining. To detect binding of PR3 to HUVEC, PR3 was labeled with DIG according to the protocol provided by Boehringer Mannheim Biochemica (Mannheim, Germany). HUVEC were grown to confluence on glass coverslips coated with 1% gelatin, which had been cross-linked with 0.5% glutaraldehyde. To assess binding of PR3 to HUVEC, cells were fixed in 1% formaldehyde (10 min on ice), washed three times with PBS, and incubated with 20 $\mu\text{g}/\text{ml}$ PR3-DIG in PBS/1% bovine serum albumin (BSA) for 60 min on ice. DIG-labeled human serum albumin (HSA) and DIG-labeled monoclonal antibody (mAb) anti-CD31 (CLB, Amsterdam, The Netherlands) were used as a negative and positive control, respectively. After washing, cells were incubated with peroxidase-labeled sheep F(ab')₂ anti-DIG (60 min on ice). Cells were washed and binding was detected with tyramide-FITC and H_2O_2 (0.0005%) for 30 min on ice. The coverslips were then mounted in DABCO (1,4-diazabicyclo-(2.2.2) octane; Sigma Chemical Co., St. Louis, MO) and examined using a fluorescence microscope.

Fluorescence-Activated Cell Sorter Analysis. Fluorescence-activated cell sorter (FACS) analysis was used as one means to detect binding of PR3 to HUVEC. Briefly, HUVEC were grown to confluence and subsequently detached by incubation in PBS containing 20 mM ethylenediaminetetra-acetic acid (EDTA) (pH 7.4) for 10 min on ice, followed by 10 min at 37°C. Cells were washed twice with PBS/1% BSA. To detect binding of DIG-labeled proteins, 2×10^5 cells were incubated with various concentrations of either PR3-DIG or HSA-DIG in PBS/1% BSA for 60 min on ice. Cells were washed with PBS/1% BSA and binding was detected using mouse mAb anti-DIG DI-22 (Sigma) followed by phycoerythrin-labeled goat F(ab')₂ anti-mouse Ig (GAM-PE) (Dako, Glostrup, Denmark). Cells were washed, fixed in 1% paraformaldehyde, and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Binding was expressed as mean fluorescence intensity, as calculated by the Lysis II program.

A second method for the assessment of binding of PR3 to HUVEC used unlabeled PR3. HUVEC-bound PR3 was detected using different mouse mAb. mAb anti-PR3 4A5, 4A3, and 6A6 were a kind gift from Dr. J. Wieslander (Wieslab AB, Lund, Sweden), WGM1 and WGM2 were kindly provided by Dr. E. Csernok (Rheumaklinik Bad Bramstedt, Germany), and anti-PR3 12.8 was obtained from CLB (Amsterdam, The Netherlands). As a control, three different mAb against MPO were used: 2C7, 1H2, and 5B8 (all kind gifts from Dr. M. Audrain, Centre Hospitalier Universitaire de Nantes, France). Binding was detected using GAM-PE.

For the detection of membrane expression of PR3 by HUVEC, cells were cultured until they reached confluence and incubated with either medium alone or medium containing $\text{TNF}\alpha$ (500 U/ml) for 2 h. Possible membrane-expressed PR3 was detected by FACS as described, using different mAb directed against PR3 (4A5 or WGM2) or an isotype-matched control mAb anti-MPO (2C7), followed by GAM-PE.

The specificity of binding of PR3 was tested by incubation of

HUVEC with 5 $\mu\text{g/ml}$ PR3-DIG in the presence of increasing concentrations of unlabeled PR3. Binding was detected as described using mouse mAb anti-DIG DI-22 and GAM-PE. Using these data, the binding dissociation constant (K_d) and the number of binding sites per cell were analyzed according to the method described by van Zoelen *et al.* (16).

Cell Surface Biotinylation and Affinity Precipitation

To characterize possible membrane protein(s) to which PR3 binds, HUVEC (20×10^6 cells) were grown to confluence and detached using PBS/20 mM EDTA as described. Cells were surface-labeled with sulfo-NHS-LC-biotin (500 $\mu\text{g/ml}$; Pierce) in PBS (pH 8.0) (17). After 30 min of incubation at room temperature, the reaction was stopped by the addition of 2 mM NH_4Cl . Cells were washed twice with PBS and resuspended in 1 ml of inhibitor buffer (20 mM Hepes, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM EGTA, pH 7.5, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g/ml}$ of antipain, chymostatin, leupeptin, and pepstatin A; all from Sigma). Cells were disrupted by sonication and the membrane fraction was isolated as described by Kim *et al.* (18). The cell lysate was centrifuged at $105,000 \times g$ for 60 min, and the membrane proteins were extracted by incubation of the pellet in 0.5 ml of inhibitor buffer, supplemented with 1% Nonidet-P40 (4 h, 37°C). After centrifugation ($105,000 \times g$, 60 min), the supernatants, which contained membrane proteins, were subjected to affinity precipitation. The supernatants were precleared twice for 4 h, each time with protein G-Sepharose (20 μl ; Pharmacia Biotech). The precleared supernatants were precipitated overnight with 50 μl of protein G-Sepharose, coated with either a complex of mouse mAb anti-PR3 12.8 (200 $\mu\text{g/ml}$) and PR3 (50 $\mu\text{g/ml}$) or an isotype-matched control mAb and PR3.

The protein G pellet was washed extensively with PBS and resuspended in SDS sample buffer (New England Biolabs, Beverly, MA). Proteins were separated by SDS-PAGE (7% acrylamide gel) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking of the PVDF membranes with PBS/0.05% Tween 20/2% casein, biotinylated proteins were detected by incubation with streptavidin-horseradish peroxidase (1:100,000; Zymed, San Francisco, CA) and chemiluminescence using Supersignal Ultra (Pierce). The blots were then exposed to Hyperfilm ECL (Amersham, Little Chilton, United Kingdom).

Reverse Transcription-PCR

To assess mRNA expression of PR3 by HUVEC, HUVEC were cultured until confluence and further incubated with either medium alone or medium containing $\text{TNF}\alpha$ (500 U/ml) for 2 h. The monolayers of HUVEC were trypsinized next and the total RNA was isolated. RNA, isolated from either Jurkat or U937 cells, was used as a negative or positive control, respectively. Total cellular RNA was isolated from 1×10^6 cells using the RNazol B method (Cinna/Biotech, Houston, TX), according to the manufacturer's instructions (19). Fixed amounts of total cellular RNA (1 μg) were reverse-transcribed into cDNA by oligo(dT) priming, using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, United Kingdom). Amplification of cDNA by PCR was performed using the following primers: for PR3 forward 5'-ATCGTGGGCGGGCAGGAGGCG-3' and reverse 5'-GCGCAGCGTGGAACGGATCCA-3' and for β -actin forward 5'-CTACAATGAGCTGCGTGTGG-3' and reverse 5'-AAGGAAGGCTGGAA-GAGTGC-3', which resulted in PCR products of 662 and 527 bp, respectively.

Amplification of cDNA by PCR was performed under standard conditions (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 20 mM MgCl_2 , 0.06 mg/ml BSA, 0.25 mM dNTP, 50 pmol of each primer, and 1 U of *Taq* DNA polymerase) for 35 cycles, using 1.5 min at 95°C , 1 min at 60°C , and 3 min at 72°C (Perkin Elmer, Norwalk, CT). PCR products were analyzed on 1% agarose gels containing ethidium bromide. Results were analyzed using the Eagle Eye system (Stratagene, La Jolla, CA) and for reasons of clarity, images were black/white inverted.

Results

Binding of PR3 to HUVEC

To determine whether PR3 binds to HUVEC, PR3 was labeled with DIG, and binding to endothelial cells was assessed by indirect immunofluorescence using tyramide-FITC staining. Incubation of HUVEC with PR3-DIG (Figure 1B) showed a mild positive fluorescence of the cells compared with the DIG-labeled mAb anti-CD31 (Figure 1A), indicating binding of PR3 to HUVEC. Cells incubated with HSA-DIG only showed background staining of the nuclei (Figure 1C).

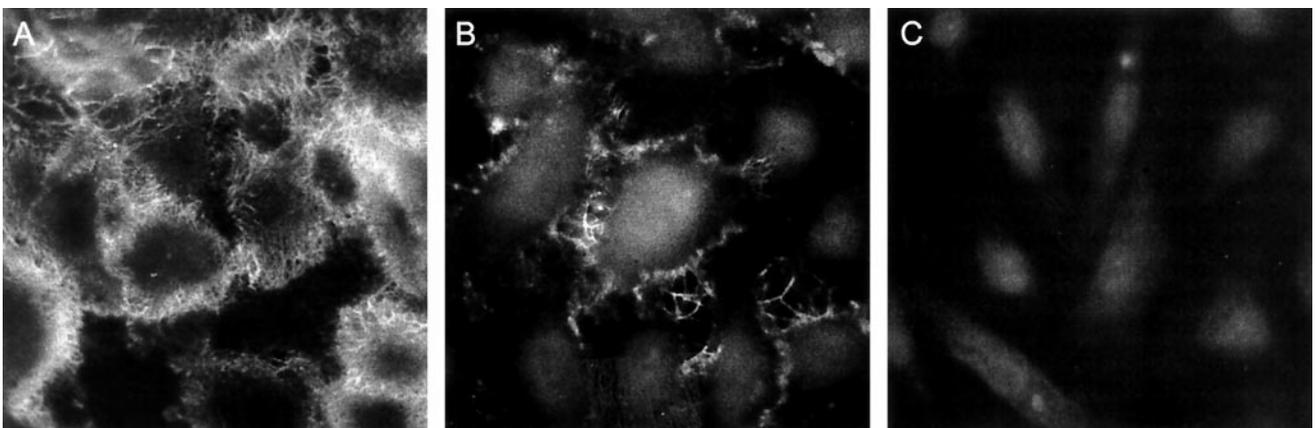


Figure 1. Visualization of binding of proteinase 3 (PR3) to human umbilical vein endothelial cells (HUVEC). HUVEC were cultured on coverslips until confluence, washed, and subsequently incubated with 20 $\mu\text{g/ml}$ PR3-digoxigenin (DIG) (B), followed by peroxidase-labeled sheep F(ab')_2 anti-DIG. Binding was detected with tyramide-FITC. As a positive control, cells were incubated with a DIG-labeled monoclonal antibody (mAb) anti-CD31 (A), whereas incubation of HUVEC with human serum albumin (HSA)-DIG served as a negative control (C). Magnification, $\times 400$.

Binding of PR3 to HUVEC was also assessed by FACS using either PR3-DIG or unlabeled PR3, the latter being detected with different mAb anti-PR3. Incubation of HUVEC with 20 $\mu\text{g/ml}$ PR3-DIG resulted in a clear positive fluorescence compared with incubation of the cells with HSA-DIG (Figure 2A). HSA-DIG exhibited only a low background binding compared with PBS. Twenty of 20 primary HUVEC cultures tested for binding of PR3 were positive. Binding of PR3 to HUVEC could be detected from passage 2 up to passage 8.

Later passages of cells were not analyzed. For FACS analysis, it was necessary to detach HUVEC with PBS containing 20 mM EDTA, because detachment with trypsin or dispase resulted in a strong reduction of PR3 binding (data not shown).

Different mAb directed against PR3 were used to detect binding of unlabeled PR3 by an indirect method. Again, binding of PR3 to HUVEC was clearly positive, when detected with anti-PR3 mAb 4A5 (Figure 2B), whereas the isotype-matched control mAb 2C7 showed no significant binding to HUVEC after incubation with PR3 (Figure 2C). In the absence of PR3, no binding of either mAb 4A5 or 2C7 could be detected. Binding of PR3 to HUVEC could be detected by different anti-PR3 mAb, although the fluorescence intensity varied between mAb (Table 1). None of the anti-MPO mAb exhibited any reactivity with HUVEC.

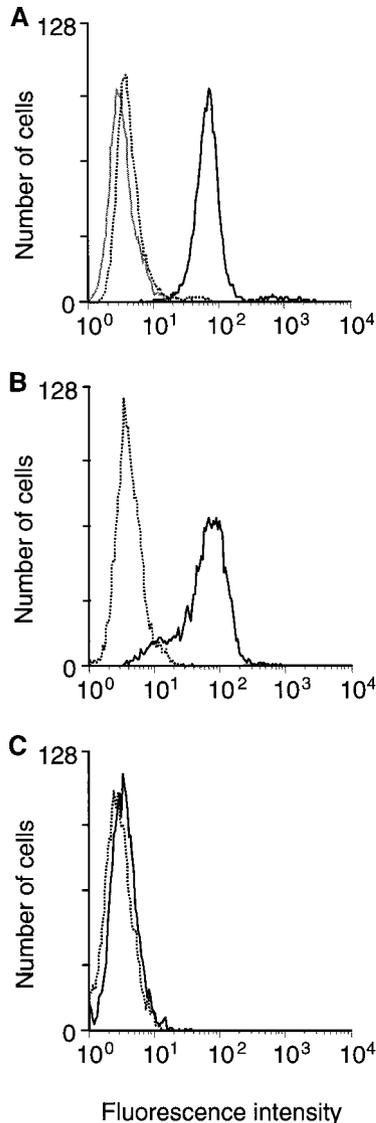


Figure 2. Analysis of binding of PR3 to HUVEC by fluorescence-activated cell sorter analysis (FACS). (A) HUVEC were incubated with HSA-DIG (20 $\mu\text{g/ml}$) (dotted line), phosphate-buffered saline (PBS) (gray solid line), or PR3-DIG (black solid line). Binding was detected with a mAb anti-DIG and phycoerythrin-labeled goat F(ab')₂ anti-mouse Ig (GAM-PE). (B and C) Indirect detection of binding of native PR3 to HUVEC using mAb, followed by detection with GAM-PE. HUVEC were incubated in the absence (dotted line) or presence (solid line) of PR3 (20 $\mu\text{g/ml}$), and binding was detected with mAb anti-PR3 4A5 (B) or an isotype-matched control mAb anti-myeloperoxidase, 2C7 (C).

Expression of PR3 by HUVEC

Our results, in contrast to earlier reports (20), suggest strongly that PR3 is not expressed by resting endothelial cells. To further clarify this issue, confluent layers of HUVEC were cultured in either medium alone or in medium containing TNF α (500 U/ml) for 2 h and washed, and then surface expression of PR3 was analyzed by FACS. No PR3 expression could be detected on either nonstimulated or on TNF α -stimulated HUVEC using either mAb WGM2 or 4A5 (Figure 3). However, exposure of HUVEC to PR3 (20 $\mu\text{g/ml}$) and subsequent detection with either mAb WGM2 or 4A5 resulted in positive fluorescence. Three primary HUVEC cultures were tested and none of them showed membrane expression of PR3, either unstimulated or after stimulation with TNF α for 2 h

Table 1. Binding of PR3 to HUVEC as assessed by FACS analysis using different mAb^a

mAb	Isotype	MFI	
		PBS	PR3
mAb-α-PR3			
4A5	IgG ₁	4.7	67.4
4A3	IgG _{2a}	4.8	127.1
6A6	IgG ₁	3.0	44.8
12.8	IgG ₁	3.9	61.4
WGM1	IgM	7.0	18.1
WGM2	IgG ₁	3.6	11.2
mAb-α-MPO			
2C7	IgG ₁	3.6	4.2
1H2	IgG _{2b}	4.9	6.2
5B8	IgG ₁	8.3	9.6

^a HUVEC were incubated in the absence or presence of PR3 (20 $\mu\text{g/ml}$), and binding was detected using different mAb directed against PR3, followed by GAM-PE. mAb anti-MPO served as a control. Binding of the mAb was expressed as mean fluorescence intensity (MFI). PR3, proteinase 3; HUVEC, human umbilical vein endothelial cells; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GAM-PE, phycoerythrin-labeled goat F(ab')₂ anti-mouse Ig; MPO, myeloperoxidase.

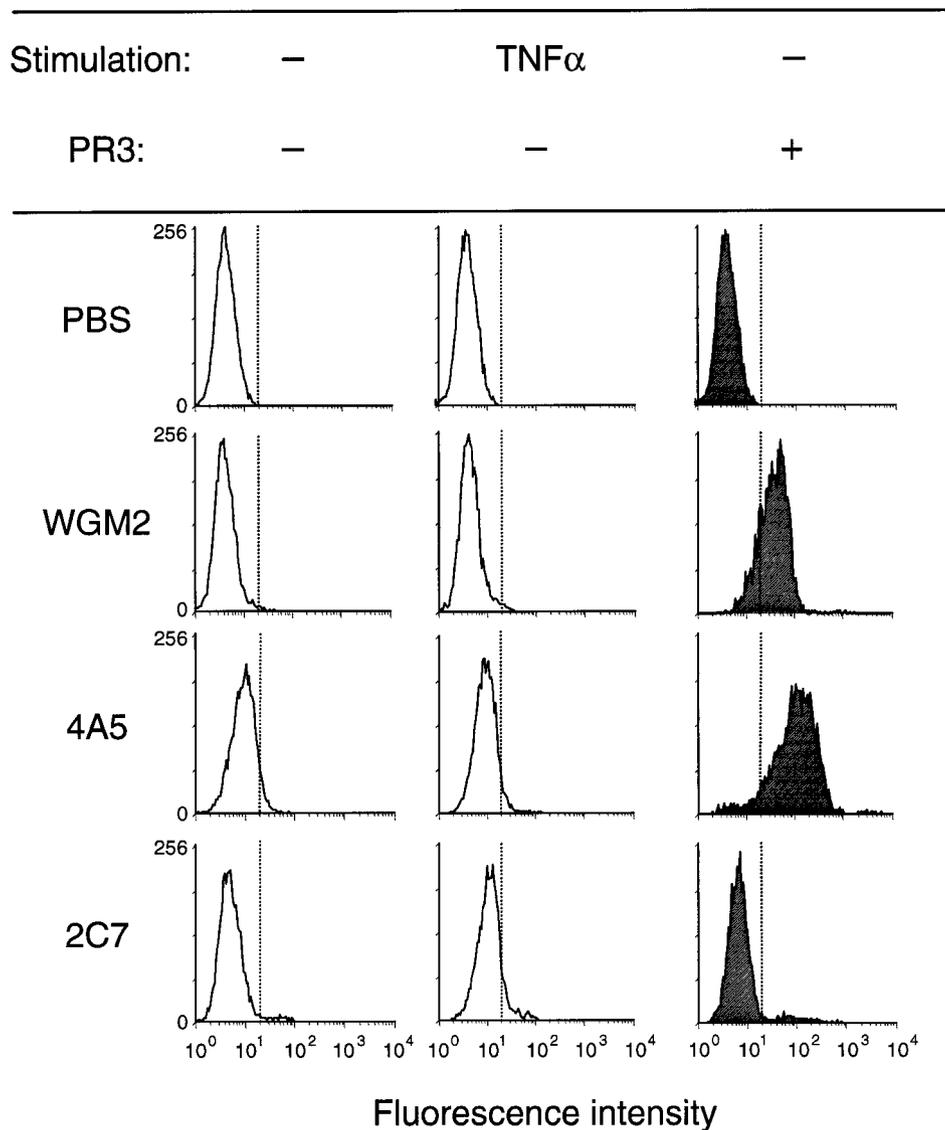


Figure 3. Membrane expression of endogenous PR3 by HUVEC as detected by FACS. Cells were stimulated with medium alone or medium containing tumor necrosis factor- α (TNF α ; 500 U/ml) for 2 h, and membrane expression of PR3 was assessed using the anti-PR3 mAb WGM2 and 4A5. mAb 2C7 served as an isotype-matched control.

Table 2. Absence of endogenous surface expression of PR3 on HUVEC as assessed by FACS analysis^a

HUVEC	Unstimulated			TNF α -Stimulated		
	WGM2	4A5	2C7	WGM2	4A5	2C7
1	2.4	2.2	2.5	2.7	3	2.4
2	2.2	4.6	2.9	2.8	4.5	3.6
3	5.3	12.5	9.0	5.7	11.9	16.1

^a HUVEC obtained from three different primary cultures were stimulated with medium alone or medium containing TNF α (500 U/ml) for 2 h, and surface expression of PR3 was assessed using two anti-PR3 mAb (WGM2 and 4A5), while 2C7 served as an isotype-matched control. Data are expressed as mean fluorescence intensity (MFI). TNF α , tumor necrosis factor- α . Other abbreviations as in Table 1.

(Table 2). The absence of endogenous membrane expression of PR3 on HUVEC was confirmed at the mRNA level. HUVEC were stimulated with medium alone or medium containing TNF α (500 U/ml) for 2 h, and total RNA was isolated and

analyzed for expression of PR3 mRNA by reverse transcription (RT)-PCR. None of the three primary HUVEC cultures showed expression of PR3 mRNA, either unstimulated or after TNF α stimulation (Figure 4). RT-PCR for PR3 expression in

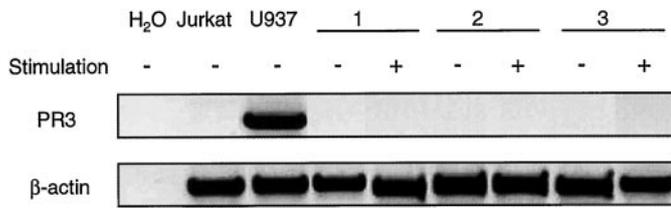


Figure 4. Reverse transcription (RT)-PCR analysis to detect PR3 mRNA expression. Expression of PR3 mRNA by three different primary HUVEC cultures was assessed by RT-PCR after stimulation of the cells with medium alone or medium containing TNF α (500 U/ml) for 2 h. Jurkat and U937 cells served as negative and positive controls, respectively.

U937 cells, cells that were used as a positive control, showed a band of the expected size (662 bp), whereas Jurkat cells were negative.

Dose-Dependent Binding of PR3 to HUVEC

Incubation of HUVEC with increasing concentrations of PR3-DIG resulted in dose-dependent binding of the ligand as detected by FACS analysis (Figure 5). Binding of PR3 was already detectable at a concentration of 1.25 μ g/ml PR3-DIG, and saturation of binding was reached at a concentration of 40 μ g/ml. HSA-DIG only showed weak background binding.

To determine the specificity and affinity of binding of PR3 to endothelial cells, HUVEC were incubated with a constant quantity of PR3-DIG (5 μ g/ml) in the presence of increasing concentrations of unlabeled PR3. A dose-dependent inhibition of binding of PR3-DIG was observed, reaching plateau levels of inhibition in the presence of a 71 molar excess of unlabeled PR3 (Figure 6). A maximum inhibition of binding of 89% was found. Higher concentrations of PR3 resulted in cytolysis. Analysis of the data according to van Zoelen *et al.* (16) resulted in a calculation of a K_d of 0.1 μ M for binding of PR3 to HUVEC and 2000 ± 475 PR3 binding sites per endothelial cell.

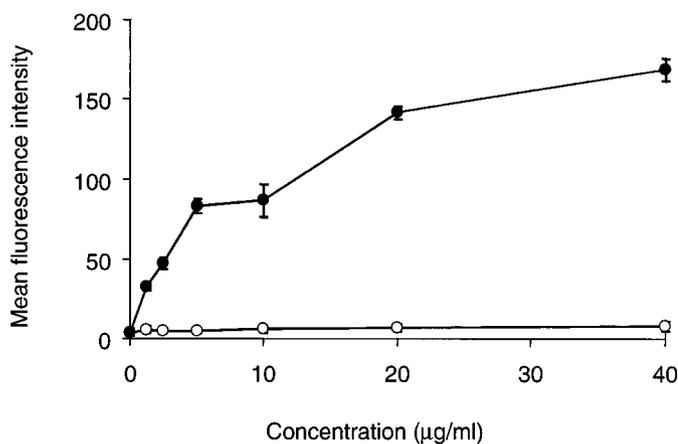


Figure 5. Dose-dependent binding of DIG-labeled PR3 to HUVEC as assessed by FACS analysis. HUVEC were incubated with increasing concentrations of either PR3-DIG (filled circles) or HSA-DIG (open circles), and binding was detected by mAb anti-DIG and GAM-PE.

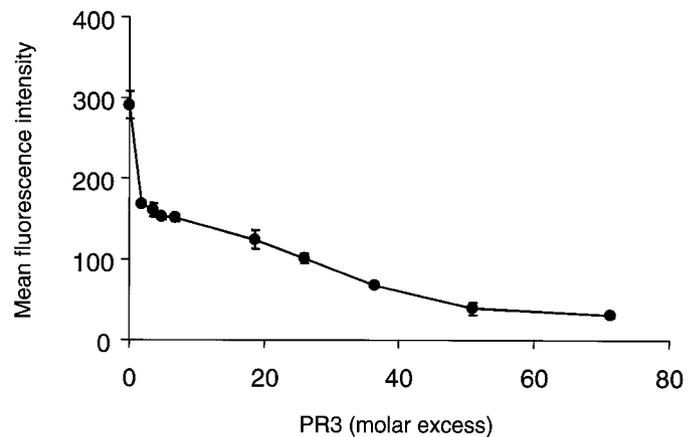


Figure 6. Inhibition of binding of PR3-DIG to HUVEC with unlabeled PR3, as assessed by FACS. HUVEC were incubated with PR3-DIG (5 μ g/ml) in the presence of increasing concentrations of unlabeled PR3. Binding of PR3-DIG to HUVEC was detected with mAb anti-DIG and GAM-PE.

Characterization of Potential PR3-Binding Membrane Molecules

To characterize the membrane proteins on endothelial cells, which bind PR3, HUVEC were surface-labeled with biotin and lysed, and the membrane proteins were extracted. PR3-specific membrane proteins were precipitated with protein G-Sepharose, which had been coated with a complex of mAb 12.8 and PR3 and subsequently analyzed by SDS-PAGE and Western blotting. Analysis of the precipitated proteins revealed a band with an apparent molecular weight of 111 kD under nonreducing conditions and bands of 52 and 63 kD under reducing conditions (Figure 7). Control precipitation of membrane proteins with protein G-Sepharose, which had been coated with a complex of the isotype-matched control mAb anti-MPO, 5B8, and PR3, resulted in lightly stained bands at 129 and 77 kD, which were different from the one precipitated with mAb 12.8 and PR3.

Discussion

This is the first report to demonstrate specific binding of the neutrophil serine proteinase PR3 to human endothelial cells. A previous report mentioned binding of PR3 to HUVEC after incubation of HUVEC with PR3 and subsequent detection with six cytoplasmic ANCA-positive sera (21). These authors suggested that deposition of PR3 on endothelial cells occurred through charge interactions. Several *in vitro* studies have suggested a role for PR3 in endothelial cell injury in Wegener's granulomatosis, either by causing endothelial cell detachment and cytolysis (7) or by apoptosis of endothelial cells (8,9). Furthermore, PR3 may play a direct role in endothelial cell activation by enhancing IL-8 production (10). This latter cell activation was observed using either enzymatically active or inactive PR3, suggesting direct binding of PR3 to endothelial cells.

Indeed, incubation of HUVEC with PR3-DIG resulted in positive binding, as demonstrated by indirect immunofluores-

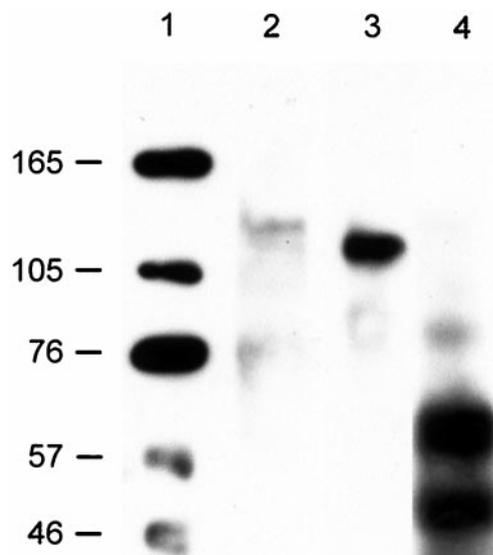


Figure 7. Characterization of PR3-binding membrane molecules on HUVEC. HUVEC membrane proteins were precipitated with protein G-Sepharose, which had been coated with a complex of mAb 12.8 and PR3 (lanes 3 and 4) or an isotype-matched control mAb 5B8 and PR3 (lane 2) and subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot. Gel electrophoresis was performed under nonreducing conditions (lanes 2 and 3) or reducing conditions (lane 4). To visualize the bands in lane 4 more clearly, the exposure time of this lane was four times as long as that of lanes 2 and 3. Lane 1 contains molecular weight markers of the indicated molecular weights in kilodaltons.

cence using HUVEC cultured on coverslips. In this assay, it was necessary to fix the cells lightly with 1% formaldehyde. To assess binding of PR3 to nonfixed HUVEC and to obtain a more quantitative assessment of binding, FACS analysis was performed. Again, we demonstrated binding of PR3 to HUVEC, using either DIG-labeled PR3 or unlabeled PR3. Several mAb directed against PR3 were used to reveal binding of native PR3 to HUVEC. All mAb recognized exogenously added PR3 bound to the cell surface as shown by increased fluorescence intensity. However, differences in the increase of fluorescence intensity were observed between the different mAb to PR3, a feature that may be explained by the recognition of different epitopes on PR3 by these mAb.

To test the possibility that a specific binding site on endothelial cells was involved in increased FACS fluorescence, we studied the binding characteristics of PR3 to HUVEC in more detail. PR3 was found to bind to HUVEC dose-dependently, reaching saturation at a concentration of 40 $\mu\text{g/ml}$. Because this result is compatible with binding of PR3 to a receptor, we tested the specificity of binding. Incubation of HUVEC with PR3-DIG in the presence of increasing concentrations of unlabeled PR3 resulted in a dose-dependent inhibition of binding, reaching a maximum inhibition of 89%. Higher concentrations of unlabeled PR3 resulted in cytolysis and could not be analyzed. Analysis of our data revealed that PR3 binds to HUVEC with a K_d of 0.1 μM . Interaction of PR3 with reconstituted lipid bilayers showed partial insertion of PR3 into the hydro-

phobic region of the lipid bilayer with a K_d of 4.5 μM (22). However, a different mechanism of interaction may be involved in this latter case, since in this study PR3 interacts with lipids, whereas in our study PR3 interacts with a membrane protein.

Indeed, detachment of the cells with either trypsin or dispase resulted in a strong reduction in binding of PR3 to HUVEC, as detected by FACS analysis, results which suggest that a protein present on endothelial cells might be involved in the binding of PR3. Affinity precipitation of HUVEC membrane proteins with protein G-Sepharose, coated with a complex of mAb 12.8 and PR3, revealed a band with a molecular weight of 111 kD under nonreducing conditions and bands of 52 and 63 kD under reducing conditions. These results suggest that the PR3 binding protein may be a heterodimer, composed of two proteins. Protease-activated receptors (PAR) (23), which are expressed by endothelial cells and are activated by proteases through cleavage of the protein's N terminus, might be possible candidate receptors for binding of PR3. Cathepsin G has been shown to cleave the thrombin receptor (PAR-1) at a site distal from thrombin, thus disabling the receptor for subsequent activation by thrombin. However, if this cleavage site is somehow protected, cathepsin G may activate PAR-1 (24). Cathepsin G was found to neither activate nor disable PAR-2 (25), and both cathepsin G and neutrophil elastase were found to have no effect on PAR-3 (26). For PR3, no effects on the activation of PAR have been described thus far. Thrombin has been reported to stimulate IL-8 production by HUVEC through catalytic activation of the thrombin receptor (27). PR3 also enhanced IL-8 production by HUVEC, but independent of its enzymatic activity (10), suggesting a different mechanism of endothelial cell activation in this case. Furthermore, PAR-1 belongs to the family of G protein-coupled receptors and is comprised of a single polypeptide chain of approximately 60 to 62 kD (28), whereas PR3 precipitated a protein of 111 kD, which was comprised of two subunits. Further identification of the PR3 binding molecule is currently under investigation.

There are at least three possible mechanisms by which PR3 and ANCA may be involved in the pathogenesis of vasculitis. One hypothesis claims a role for ANCA by stimulating neutrophil cytotoxicity toward cultured endothelial cells (29). Priming of neutrophils with low doses of TNF and IL-8 induces partial degranulation with translocation of PR3 to the cell surface, where it becomes accessible to binding by anti-PR3 antibodies (30). Subsequent binding of ANCA results in activation of the cytokine-primed PMN (2,3). ANCA may also activate PMN via Fc γ RII (31,32). Activation of PMN may lead to a respiratory burst with production of reactive oxygen radicals and cellular degranulation, resulting in release of proteolytic enzymes (2,3,33) and subsequent endothelial cell injury. Recently, PR3 was shown to enhance the release of TNF α and IL-1 β from a human monocytic cell line that had been stimulated with lipopolysaccharide and *N*-formyl-Nle-Leu-Phe (11), suggesting a possible indirect route of endothelial cell activation by PR3. A third mechanism of endothelial cell activation was described by Mayet *et al.* (20), who showed that HUVEC produce PR3. These authors observed that activation of endo-

thelial cells with cytokines, such as TNF α or IL-1 α , induced a translocation of PR3 from the perinuclear region to the cytoplasm and a transient expression of PR3 on the cell surface. Thus, PR3 may become accessible to binding by ANCA and lead to endothelial cell activation. In subsequent experiments, increased neutrophil adhesion to endothelial cells and induction of endothelial E-selectin expression (34) and vascular cell adhesion molecule-1 expression (35) followed incubation of HUVEC with purified anti-PR3 antibodies. Priming of endothelial cells with TNF α for 2 h, followed by challenge with anti-PR3 antibodies, led to prostacyclin and platelet-activating factor synthesis (36). However, synthesis of PR3 by endothelial cells is a controversial issue. King *et al.* (37) found no evidence that endothelial cells produce PR3 or express PR3 on the cell surface. To exclude the possibility that we detected endogenously produced PR3, we studied the surface expression of PR3. No membrane expression of PR3 could be detected by FACS analysis using two different anti-PR3 mAb, either on unstimulated or TNF α -stimulated HUVEC. In addition, RT-PCR analysis gave no proof for production of PR3 mRNA by HUVEC. Furthermore, addition of exogenous PR3 resulted in a strong positive fluorescence as detected by FACS analysis, indicating that only binding of exogenous PR3 to HUVEC was detected in our assays.

Because PR3 was shown to enhance IL-8 production by HUVEC, we hypothesize that PR3 may directly interact with endothelial cells. PR3 may be released systemically or locally at inflammatory sites after activation of primed PMN (2,3) and may directly contribute to endothelial cell injury and/or activation of endothelial cells. Subsequent binding of ANCA to endothelial cell-bound PR3 may then lead to further amplification of an inflammatory cascade. In this study, we have shown a dose-dependent, specific, and saturable binding of PR3 to HUVEC. This binding involves a membrane-binding protein with a molecular weight of 111 kD.

Acknowledgments

The investigations reported here were supported by Grant C95.1458 from the Dutch Kidney Foundation. We thank Dr. R. Wes Leid for critically reading the manuscript.

References

- Godman GC, Churg J: Wegener's granulomatosis: Pathology and review of the literature. *Arch Pathol* 58: 533–552, 1954
- Falk RJ, Terrell RS, Charles LA, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA* 87: 4115–4119, 1990
- Keogan MT, Esnault VL, Green AJ, Lockwood CM, Brown DL: Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. *Clin Exp Immunol* 90: 228–234, 1992
- Baslund B, Petersen J, Permin H, Wiik A, Wieslander J: Measurement of proteinase 3 and its complexes with α_1 -proteinase inhibitor and anti-neutrophil cytoplasm antibodies (ANCA) in plasma. *J Immunol Methods* 175: 215–225, 1994
- Henshaw TJ, Malone CC, Gabay JE, Williams RC Jr: Elevations of neutrophil proteinase 3 in serum of patients with Wegener's granulomatosis and polyarteritis nodosa. *Arthritis Rheum* 37: 104–112, 1994
- Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR: Proteinase 3: A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. *J Clin Invest* 82: 1963–1973, 1988
- Ballieux BE, Hiemstra PS, Klar-Mohamad N, Hagen EC, van Es LA, van der Woude FJ, Daha MR: Detachment and cytolysis of human endothelial cells by proteinase 3. *Eur J Immunol* 24: 3211–3215, 1994
- Yang JJ, Kettritz R, Falk RJ, Jennette JC, Gaido ML: Apoptosis of endothelial cells induced by the neutrophil serine proteases proteinase 3 and elastase. *Am J Pathol* 149: 1617–1626, 1996
- Taekema-Roelvink MEJ, van Kooten C, Janssens MC, Heemskerck E, Daha MR: Effect of anti-neutrophil cytoplasmic antibodies on proteinase 3-induced apoptosis of human endothelial cells. *Scand J Immunol* 48: 37–43, 1998
- Berger SP, Seelen MAJ, Gerritsma JSJ, Heemskerck E, van der Woude FJ, Daha MR: Proteinase 3, the major autoantigen of Wegener's granulomatosis, enhances IL-8 production by endothelial cells *in vitro*. *J Am Soc Nephrol* 7: 694–701, 1996
- Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Wiczorek M, Kroona H, Leimer AH, Cheronis J: Converting enzyme-independent release of tumor necrosis factor α and IL-1 β from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci USA* 96: 6261–6266, 1999
- Leid RW, Van der Heijden I, Ballieux BEPB, Kleyburg-van der Keur C, Hagen EC, van Es LA, van der Woude FJ, Daha MR: Cleavage and inactivation of human C1-inhibitor by the human leukocyte proteinase, proteinase-3. *Eur J Immunol* 23: 2939–2945, 1993
- Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745–2750, 1973
- Miltenburg AMM, Meijer-Paape ME, Weening JJ, Daha MR, van Es LA, van der Woude FJ: Induction of antibody-dependent cellular cytotoxicity against endothelial cells by renal transplantation. *Transplantation* 48: 681–688, 1989
- Maciag T, Cerundolo J, Isley J, Kelley PR, Forand F: An endothelial growth factor from bovine hypothalamus: Identification and partial characterization. *Proc Natl Acad Sci USA* 76: 5674–5674, 1979
- van Zoelen EJJ, Kramer RH, van Reen MMM, Veerkamp JH, Ross HA: An exact general analysis of ligand binding displacement and saturation curves. *Biochemistry* 32: 6275–6280, 1993
- Gomez-Guerrero C, Duque N, Egido J: Mesangial cells possess an asialoglycoprotein receptor with affinity for immunoglobulin A. *J Am Soc Nephrol* 9: 568–576, 1998
- Kim KU, Kwon OJ, Jue DM: Pro-tumour necrosis factor cleavage enzyme in macrophage membrane/particulate fraction. *Immunology* 80: 134–139, 1993
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987
- Mayet WJ, Csernok E, Szymkowiak C, Gross WL, Meyer zum Büschenfelde KH: Human endothelial cells express proteinase 3, the target antigen of anticytoplasmic antibodies in Wegener's granulomatosis. *Blood* 82: 1221–1229, 1993

21. Savage CO, Gaskin G, Pusey CD, Pearson JD: Myeloperoxidase binds to vascular endothelial cells, is recognized by ANCA and can enhance complement dependent cytotoxicity. *Adv Exp Med Biol* 336: 121–123, 1993
22. Goldmann WH, Niles JL, Arnaout MA: Interaction of purified human proteinase 3 (PR3) with reconstituted lipid bilayers. *Eur J Biochem* 261: 155–162, 1999
23. Cupit LD, Schmidt VA, Bahou WF: Proteolytically activated receptor-3: A member of an emerging gene family of protease receptors expressed on vascular endothelial cells and platelets. *Trends Cardiovasc Med* 9: 42–48, 1999
24. Molino M, Blanchard N, Belmonte E, Tarver AP, Abrams C, Hoxie JA, Cerletti C, Brass LF: Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. *J Biol Chem* 270: 11168–11175, 1995
25. Molino M, Woolkalis MJ, Reavy-Cantwell J, Pratico D, Andrade-Gordon P, Barnathan ES, Brass LF: Endothelial cell thrombin receptors and PAR-2: Two protease-activated receptors located in a single cellular environment. *J Biol Chem* 272: 11133–11141, 1997
26. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR: Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 386: 502–506, 1997
27. Ueno A, Murakami K, Yamanouchi K, Watanabe M, Kondo T: Thrombin stimulates production of interleukin-8 in human umbilical vein endothelial cells. *Immunology* 88: 76–81, 1996
28. Yan W, Tiruppathi C, Qiao R, Lum H, Malik AB: Tumor necrosis factor decreases thrombin receptor expression in endothelial cells. *J Cell Physiol* 166: 561–567, 1996
29. Savage COS, Pottinger BE, Gaskin G, Pusey CD, Pearson JD: Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. *Am J Pathol* 141: 335–342, 1992
30. Csernok E, Ernst M, Schmitt W, Bainton DF, Gross WL: Activated neutrophils express proteinase 3 on their plasma membrane *in vitro* and *in vivo*. *Clin Exp Immunol* 95: 244–250, 1994
31. Porges AJ, Redecha PB, Kimberly WT, Csernok E, Gross WL, Kimberly RP: Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc γ RIIa. *J Immunol* 153: 1271–1280, 1994
32. Mulder AHL, Heeringa P, Brouwer E, Limburg PC, Kallenberg CGM: Activation of granulocytes by anti-neutrophil cytoplasmic antibodies: A Fc- γ -RII-dependent process. *Clin Exp Immunol* 98: 270–278, 1994
33. Charles LA, Caldas ML, Falk RJ, Terrell RS, Jennette JC: Antibodies against granule proteins activate neutrophils *in vitro*. *J Leukocyte Biol* 50: 539–546, 1991
34. Mayet WJ, Meyer zum Büschenfelde KH: Antibodies to proteinase 3 increase adhesion of neutrophils to human endothelial cells. *Clin Exp Immunol* 94: 440–446, 1993
35. Mayet WJ, Schwarting A, Orth T, Duchman R, Meyer zum Büschenfelde KH: Antibodies to proteinase 3 mediate expression of vascular cell adhesion molecule-1 (VCAM-1). *Clin Exp Immunol* 103: 259–267, 1996
36. Sibelius U, Hattar K, Schenkel A, Noll T, Csernok E, Gross WL, Mayet WJ, Piper H, Seeger W, Grimminger F: Wegener's granulomatosis: Anti-proteinase 3 antibodies are potent inducers of human endothelial cell signalling and leakage response. *J Exp Med* 187: 497–503, 1998
37. King WJ, Adu D, Daha MR, Brooks CJ, Radford DJ, Pall AA, Savage CO: Endothelial cells and renal epithelial cells do not express the Wegener's autoantigen, proteinase 3. *Clin Exp Immunol* 102: 98–105, 1995