Human Endothelial Cells Express Proteinase 3, the Target Antigen of Anticytoplasmic Antibodies in Wegener’s Granulomatosis


Autoantibodies directed against cytoplasmic antigens of neutrophils (ANCA), especially proteinase 3 (PR-3), have proved to be a useful clinical tool confirming the diagnosis of Wegener’s granulomatosis (WG). Although several concepts concerning the pathophysiologic potentials of ANCA have been discussed, only sparse data about ANCA-endothelium interactions have been available. In this study, we have investigated the expression of PR-3 in cytokine-treated human endothelial cells using purified anti-PR-3 antibodies of patients with WG, murine and human monoclonal anti-PR-3 antibodies as probes. We were able to show that tumor necrosis factor-α, interleukin-1α/β, and interferon-γ led to an increased PR-3 expression in the cytoplasm of endothelial cells by performing polymerase chain reaction analysis. Western blot, cyto–enzyme-linked immunosorbent assays, and confocal laser scanning microscopy. Moreover, PR-3 was also translocated into the cell membrane, becoming accessible to ANCA. Our data suggest a possible direct pathogenic effect of anti-PR-3 antibodies in WG and other vasculitides. Anti-PR-3 antibodies represent an important missing link in ANCA-endothelial interactions.

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ANTINEUTROPHIL cytoplasmic autoantibodies (ANCA) are useful diagnostic serologic markers for a variety of well-known vasculitic syndromes, including Wegener’s granulomatosis (WG), polyarteritis nodosa, Churg Strauss syndrome, and pulmonary-renal syndrome with alveolar capillaritis.

Using the indirect immunofluorescence technique (IFT), two different ANCA types have been described. One ANCA type shows cytoplasmic staining (C-ANCA), the other type shows a perinuclear staining (P-ANCA) and is usually specific for myeloperoxidase (MPO). Lüdemann et al. proposed that the C-ANCA antigen is identical with proteinase 3 (PR-3), a constituent of neutrophil primary granules characterized by Kao et al. and is distinct from elastase and cathepsin G. The revised PR-3 sequence suggested that this serine protease is identical to AGP7. Jenne et al. proposed that PR-3, p29, and AGP7 are identical to myeloblastin present in HL-60 human leukemic cells and are involved in the control of growth and differentiation of leukemic cells.

The association of ANCA with systemic necrotizing vasculitis and glomerulonephritis has prompted the hypothesis that these antibodies are involved in the pathogenesis of these diseases. Although several concepts concerning the pathophysiologic potentials of ANCA have been discussed, until now only sparse data about ANCA-endothelium interactions have been available.

ANCA could be directed against endothelial antigens ex-
Fig 2. (A) Indirect immunofluorescence of fixed HEC (no cytokine treatment; original magnification X 450). Only weak reaction of a purified anti-PR-3 antibody [F(ab)₂ fragments]. (B) Reaction of monoclonal anti-PR-3 antibody WGM2 on fixed HEC after 30 minutes of treatment with TNF-α (immungold staining; original magnification X 1,000). Diffuse cytoplasmic reaction. (C) Indirect immunofluorescence of fixed HEC after 30 minutes of treatment with TNF-α (original magnification X 450). Reaction of a purified anti-PR-3 antibody [F(ab)₂ fragments] with diffuse cytoplasmic staining.
pressed as a consequence of vascular damage, and thus represent markers of endothelial dysfunction. We were recently able to detect PR-3 in cytokine-treated human tumor cells, therefore refuting the exclusivity of neutrophils and monocytes as the sole source for this antigen. These findings were the impetus to investigate a possible expression of PR-3 in human endothelial cells, which could represent one important prerequisite of ANCA-endothelium interactions.

PATIENTS AND METHODS

**Serum samples.** Serum samples were obtained from 150 donors. Fifty donors suffered from clinically active WG. The diagnosis was established on the basis of classical symptoms and the typical histologic findings in biopsy specimens, as described earlier. One hundred healthy blood donors (HBD) served as controls.

**Antibody testing.** All WG sera were tested for anti–PR-3 antibodies by IFT on fixed neutrophils, enzyme-linked immunosorbent assay (ELISA), and Western blot. Several antigen preparations served as antigens: α-extract of human neutrophils, purified PR-3, MPO, cathepsin G, and elastase. ELISAs were performed as described earlier. Polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed as described previously. In addition, all sera were screened with routine methods for other antibody specificities. Rheumatoid factor (RF) was determined by a latex fixation test (Behring Werke Marburg, Marburg, Germany).

**Purification of anti–PR-3 antibodies.** IgG was prepared from 10 monospecific anti–PR-3 antibody-positive WG sera by ammonium sulfate precipitation and ion exchange chromatography on diethylaminoethyl (DEAE)-Sephadex (Pharmacia, Uppsala, Sweden). Anti–PR-3 antibodies were affinity-purified as described earlier using purified PR-3. F(ab′)2 fragments were prepared as described earlier. For inhibition experiments, serum antibodies were mixed with affinity-purified antigen and PR-3 purified as described by Kao et al., vol/vol diluted to 0.1 mg/mL protein concentration in phosphate-buffered saline (PBS), and incubated on a rotator for 1 hour at 37°C and 12 hours at 4°C. The mixture was centrifuged at 30,000g for 15 minutes at 4°C and the supernatants kept as absorbed material.

An anti–PR-3–specific B-cell clone (IgG) has been established and characterized earlier. In addition, a murine monoclonal antibody to PR-3 (WGM2) served as control. Isolation and culture of human endothelium cells. Human umbilical cord endothelial cells (HEC) were isolated according to the method of Jaffe et al. and cultured under standard conditions. These cells were used for further experiments between passages 4 and 6. Cells of 10 donors were pooled to exclude the influence of blood group antigens.

**Fig 3.** PAGE and Western Blot with affinity-purified PR-3 using an extract of cytokine-treated HEC. (A) Gel with purified PR-3 at 29 Kd. (B) Blot. Reaction of anti–PR-3 antibody Ho3, purified anti–PR-3 antibodies (2 X), and a C-ANCA + serum (1:160) of a patient with WG at 29 Kd. (C) Blot. Serum of healthy blood donor. No reaction.

**Fig 4.** Confocal laser scanning microscopy of TNF-α–treated fixed HEC (original magnification X 630). Reaction of a purified anti–PR-3 antibody (Fab′)2 fragments. (A) Perinuclear distribution of the antigen after 5 minutes of cytokine treatment (n = nucleus). (B) Binding in the Golgi region after 10 minutes of cytokine treatment.
Fig 5. Confocal laser scanning microscopy of TNF-α-treated fixed HEC (original magnification × 630). Reaction of a purified anti-PR-3 antibody [F(ab)₂ fragments]. (A) Binding in the endoplasmic reticulum after 20 minutes of cytokine treatment (n = nucleus). (B) Cytoplasmic distribution of PR-3 after 30 minutes of cytokine treatment.

Contrast light microscopy showing the typical cobblestone monolayer appearance of cells. The purity of culture was tested with antibodies to von Willebrand’s factor and Ulex lectine. HEC were passaged on gelatine-coated culture slides (Lab-Tek; Miles Scientific, Naperville, IL) or Primaria culture dishes (Falcon, NJ) and fixed with ethanol 96%, methanol (abs.), or paraformaldehyde 3.7% (−20°C for 10 minutes). Tumor necrosis factor-α (TNF-α) (3 ng/mL; Boehringer Mannheim, Mannheim, Germany), interleukin-1α (IL-1α) (10 U/mL; Sigma, Deisenhofen, Germany), and interferon-γ (IFN-γ) (100 U/mL; Sigma) were added to the medium before fixation to test the influence of different cytokines on antigen expression. Appropriate cytokine concentrations were determined by testing dilutions from 0.001 to 100 ng/mL (TNF-α), 0.001 to 100 U/mL (IL-1α), and 0.01 to 1,000 U/mL (IFN-γ). A polyclonal anti-TNF antibody (Camon, Wiesbaden, Germany) was used to block TNF-specific effects in some tests. To determine whether expression of PR-3 is dependent on protein synthesis, effects of cycloheximide (10 μg/mL; C6255 Sigma) were also investigated. Cells on Primaria culture dishes were left unfixed. Cells were incubated with WG sera, purified IgG/F(ab)₂ fragments, the supernatant of the PR-3-specific B-cell clone Ho3, and WGM2 diluted 1/40 in PBS for 1 to 120 minutes in a humid chamber at room temperature or 4°C. After extensive washing with PBS, the cells were incubated with the second antibody, fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (F-5512; Sigma). Some experiments were also performed with immunogold staining (Biotin/avidine system with silver enhancement; Aurion, Frankfurt, Germany). Sections were incubated with Mowiol (Mowiol 4-88, Höchst, Germany) after another washing step. For detecting surface expression of antigens, all experiments were performed with unfixed live cells, as described earlier. Stained cells were analyzed with a Zeiss Axiophot microscope and a Zeiss LSM 10 laser scan microscope (Zeiss, Oberkochen, Germany), as described earlier.

Cyto-ELISAs with unfixed cytokine-treated HEC were performed as described by Frampton et al. with minor modifications. To determine specific antibody binding, experiments were performed with HEC preincubated with heat-aggregated human IgG to block Fc-receptors and with immune complex-depleted sera. For removal of immune complexes, some sera were centrifuged at 100,000g (1 hour).

Preparation of cell extracts. An extract (α-fraction) of human neutrophils was prepared as described by Rasmussen et al. and Savage et al. (‘acid’ extract). An extract of HEp2 cells was prepared as described earlier. Total extracts of cytokine-treated HEC were prepared according to Albeda et al. Only cell cultures free of fibroblasts or monocytes obtained after several passages were used for these experiments. Cells were confirmed to be HEC by morphologic criteria and by IFT with antibodies to von Willebrand’s factor. Purification of PR-3. PR-3 was purified as described by Kao et al. and affinity purified as described by Lüdemann et al. using an extract of granulocytes or cytokine-treated HEC.

RNA isolation and reverse transcription. RNA was isolated from 10⁶ cells using the method described by Chomczynski and Sacchi with RNAzol B (Cinna/Biotex, Friendswood, TX) according to the manufacturer’s instructions. RNA was treated with RNase-free DNAse (Ambion, Austin, TX) to remove contaminating DNA. Hydrolisis products were precipitated with ethanol and dissolved in water.

Fig 6. Visualization of RT-PCR products by chemiluminescence. Lane L, DNA-ladder biotinylated fX174 DNA digested with HinfI. Lane 1, endothelial cells stimulated with TNF-α. Lane 2, endothelial cells unstimulated. Lane 3, HL-60 cells (control). Lane 4, WGH1 cells (control). Lane 5, mouse Ag8 cells (negative control). Lane 6, plasmid DNA PR-3. Lane 7, minus DNA PCR control. Lanes 3, 4, and 6 are positive controls.
Fig 7. Cyto-ELISA with unfixed HEC. Measurements at 0, 1, 2, 4, and 24 hours of treatment of cells with TNF-α. One mark represents the mean of three measurements. WG 1-10, affinity-purified anti-PR-3 antibodies of 10 sera of patients with WG. PR3-EBV, anti-PR-3 antibody Ho3. NS-Pool, pool of 100 healthy blood donors. Peak-surface expression of PR-3 after 2 hours of TNF-α treatment.

Fig 7. Cyto-ELISA with unfixed HEC. Measurements at 0, 1, 2, 4, and 24 hours of treatment of cells with TNF-α. One mark represents the mean of three measurements. WG 1-10, affinity-purified anti-PR-3 antibodies of 10 sera of patients with WG. PR3-EBV, anti-PR-3 antibody Ho3. NS-Pool, pool of 100 healthy blood donors. Peak-surface expression of PR-3 after 2 hours of TNF-α treatment.

To the manufacturer's protocol and dissolved in 100 μL DEPC H2O. mRNA was extracted using 50 μL Dynabead oligo (dT)25 (Dynal AS, Oslo, Norway) according to the manufacturer's protocol. The mRNA was used in a 40-μL reverse transcriptase reaction (50 mmol/L Tris-HCl, pH 8.3, 40 mmol/L KCl, 1 mmol/L DTT, 6 mmol/L MgCl2, 1 mmol/L rNTPs, 0.5 μg oligo [dT]12-18, 0.1 mg/mL BSA), 18 U RNAguard (Pharmacia-LKB, Uppsala, Sweden) with 200 U MMLV reverse transcriptase (GIBCO-BRL, Berlin, Germany). RNA, DEPC-H2O, and oligo (dT)24 were incubated for 95°C for 5 minutes and quickly chilled in ice. All other components were added and incubated at 10 minutes at room temperature and 60 minutes at 37°C. The reaction was terminated by heating to 95°C for 5 minutes and quickly chilling in ice. Twenty micrograms of Glycogen (Boehringer Mannheim) was added and the volume was increased to 100 μL with a final concentration of 0.3 mol/L NaOAc, pH 5.5. cDNA was precipitated with 1 vol 2-propanol and resuspended in 20 μL DEPC-H2O.

Polymerase chain reaction (PCR). Two microliters of the reverse transcriptase reaction was used in a 30-cycle PCR reaction (Perkin-Elmer Cetus Thermal Cycler; Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of 95°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. The reaction volume consisted of 20 μL (20 mmol/L Tris-HCl, pH 8.3, 25 mmol/L KCl, 2 mmol/L MgCl2, 0.05% Tween 20, 100 mg/mL gelatine, 20 pmol of each primer, 200 μmol/L dNTPs (Biotin-dUTP/TTP in ratio of 1:4), 0.5 U of Taq polymerase (GIBCO-BRL) and was overlayed with 50 μL mineral oil. The reaction was started after preincubation at 80°C for 10 minutes by the addition of the Taq polymerase.
Fig 8. Cyto-ELISA with unfixed HEC. Measurements at 0, 1, 2, 4, and 24 hours of treatment of cells with TNF-α. One mark represents the mean of three measurements. WG 3 IgG, IgG fraction of anti-PR-3-positive WG serum. WG 3 F(ab)₂, F(ab)₂ fragments of purified anti-PR-3 antibody after blocking of Fc receptors on endothelial cells. WG 3 depl., patient’s serum after depletion of immune complexes. WG 3 Fc-block, reaction of purified anti-PR-3 antibody after preincubation with an extract of human neutrophil granulocytes. WG 3 abs.1, reaction of anti-PR-3 antibody after preincubation with an extract of human neutrophil granulocytes. Inhibition of antibody reaction with membrane expressed PR-3. WG 3 abs.2, reaction of anti-PR-3 antibody after preincubation with purified PR-3 (Kao et al). WG 3 abs.3, reaction of anti-PR-3 antibody after preincubation with α-extract of human neutrophil granulocytes. Inhibition of antibody reaction with membrane expressed PR-3. WG 3 abs.4. reaction of anti-PR-3 antibody after preincubation with an extract of HEp2 cells. No inhibition.

Separation of DNA and visualization. Ten microliters of the PCR reaction was applied to 6% acrylamide gel (19:1) in TBE, pH 8.3 (40 mmol/L Tris-HCl, pH 8.3, 20 mmol/L Na-Borat, 1 mmol/L EDTA) and electrophoresed at 3 W constant until the BPB (Bromphenol Blue) marker had reached 14 cm. Biotin-FX174 DNA-Hinfl fragments (GIBCO-BRL) were included as molecular weight markers. The DNA was transferred to a Flash-Membran (Stratgene, La Jolla, CA) using a semidy blotter (Schleicher & Schuell, Dassel, Germany) at 150 mA for 45 minutes. The DNA was cross-linked to the membrane by UV light. The Flash detection system (Stratagene) was used for the chemiluminescent reaction and the DNA was visualized on an X-ray film.

The following primers were used: 5' primer, ATGGCCCTCCGAGATGGGCGG; 3' primer, CGGAGGACTGAGGTTGGCTGGGC.

RESULTS

Ten of 50 WG sera were exclusively positive for anti-PR-3 antibodies, as determined by IFT on human neutrophils (C-ANCA; titres, 1:310 to 1:640), by ELISAs with purified antigens, by Western blot (reaction at 29 Kd), and by other routine methods. Antibodies of these sera were purified and antibody reactivity could be blocked by incubation with purified PR-3 antigen (affinity-purified as well as Kao preparation) as measured by ELISA (shown in Fig 8 for one serum [WG3], as example) and determined by Western blot (Fig 1). Antibody reactivity could not be inhibited by preincubation with extracts of HEp2 cells or packed erythrocytes.

Affinity-purified anti-PR-3 antibodies [F(ab')₂ fragments] produced a strong diffuse cytoplasmic staining of cytokine-treated HEC (Fig 2C). Antibodies produced by the PR-3-specific B-cell clone Ho3 and the murine monoclonal antibody WGM2 showed a similar pattern (Fig 2B). In contrast, untreated cells showed only a weak reaction (Fig 2A). Modification of the fixation method had no influence on the binding pattern. A serum of a WG patient, purified PR-3 antibodies, and antibodies produced by Ho3 recognized the affinity-purified antigen (extract of TNF-α-treated HEC) at 29 Kd (Fig 3).

Cytokine treatment of HEC before fixation led to a time-dependent translocation of the antigen from the perinuclear region to the cytoplasm. Figures 4 and 5 show sequential analyses of a single experiment. Cytokines had no toxic effects on HEC as detected by trypan blue inclusion or morphologic change of cell shape. PR-3 message in HEC could be demonstrated by PCR (Fig 6).

Cytokine treatment of HEC also led to a membrane expression of PR-3. In contrast to cytoplasmic translocation, which has been investigated by IFT with fixed HEC, this was first assessed by cyto-ELISA with unfixed HEC. Figure 7 shows an increased surface expression of PR-3 under the influence of TNF-α in an ELISA with unfixed HEC. Compared with a serum pool of HBD, affinity-purified antibodies reacted rather weakly with untreated HEC (Fig 7), but TNF-α and IL-1α treatment led to a peak of antigen expres-
FIG 9. Confocal laser scanning microscopy of TNF-α-treated (120 minutes) unfixed HEC (original magnification ×630; 1 μm z-sections). Reaction of a purified anti-PR-3 antibody [F(ab'_2) fragments] with the surface expressed PR-3. (A) z = 0 μm; (B) z = 1 μm; (C) z = 2 μm; (D) z = 3 μm; (E) z = 4 μm; (F) z = 5 μm.

Discussion

WG and microscopic polyarteritis are forms of systemic vasculitis whose pathogenic mechanisms are still poorly understood. An autoimmune etiology has been implicated in WG by the finding of circulating autoantibodies shown by indirect immunofluorescence techniques to bind to cytoplasmic components of alcohol-fixed normal human neutrophils. 28

C-ANCA are seromarkers of WG and evidence exists that both the autoantigen and ANCA participate in the pathogenesis of at least the group “ANCA-associated vasculitides.” 29 Until now, concepts of a direct pathogenic effect put the neutrophil in the focus of interest. The infiltration of polymorphonuclear leukocytes has been detected in inflammatory lesions of ANCA-associated vasculitis and glomerulonephritis. 30 Several investigators report on the activation of neutrophils as important mechanism of ANCA-induced tissue destruction. 31-33 In vitro ANCA can activate neutrophils and monocytes to produce reactive oxygen species, degranulation, and endothelial cell injury. 34,35 Recently, two groups have presented a model in which ANCA and their target antigens could be involved as a major pathogenic event in vascular tissue damage. 36,37

The first contact with antigen is the luminal surface of the vascular endothelium of the tissue and this unique anatomical location of endothelial cells makes them a major candidate for initial antigen presentation. 38 Recently, Abbott et al. 39 could show by IFT that a monoclonal antibody to neutrophil cytoplasmic antigens and WG sera bound to human cultured glomerular epithelial and endothelial cells as well as to HEC. The investigators concluded that the antigen recognized by the ANCA-positive sera is also expressed on surface vascular structures, suggesting a direct pathogenic role for this antibody specificity.

These findings, together with the knowledge that neutrophils do not represent the exclusive source of PR-3, 9 were the impetus behind the investigation of the repertoire of endothelial antigens recognized by ANCA. In this study we have investigated the expression of PR-3 in HEC using purified anti-PR-3 antibodies of WG sera and monoclonal antibodies against PR-3 (murine and human) as probes.

According to our knowledge, this is the first report of PR-3 in HEC. In addition to the identification of PR-3 in untreated HEC using PCR and affinity-purified anti-PR-3 antibodies as probes, we were able to demonstrate time-dependent translocation of this antigen in the cytoplasm of HEC and a transient membrane expression after treatment with cytokines. Different cytokines showed different profiles of membrane expression. Translocation of PR-3 in HEC could be characterized as an active process depending on protein synthesis. Antibody binding does not occur unspecifically via Fc-receptors.

PR-3 under the influence of cytokines is expressed in the membrane of endothelial cells, thereby becoming accessible to circulating C-ANCA, supporting the supposition that the generation of these autoantibodies might be a fundamental process in the development of the systemic vasculitides. Thus, the ANCA antigen is present in the sites affected by the disease (ie, WG and other ANCA-related diseases).

Furthermore, our data confirm the putative key role of cytokines in ANCA-associated vasculitic syndromes. In this context, we recently have measured TNF and IL-6 levels in...
WG sera. TNF levels were elevated during active phases and not detectable in remission.37 These findings are supported by reports of enhanced transcription of TNF-α gene in peripheral blood mononuclear cells of patients with systemic vasculitis and WG compared to HBD performing RNA-dot blot or Northern blot analysis.40

In summary, our data give a hint at a possible direct pathogenic effect of anti-PR-3 antibodies in WG and represent an important missing link in ANCA-endothelial interactions. Further investigations are currently in progress to evaluate the effect of other cytokine specifities on the expression of PR-3 and to characterize cytotoxic effects of anti-PR-3 antibodies on HEC.

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Human endothelial cells express proteinase 3, the target antigen of anticytoplasmic antibodies in Wegener’s granulomatosis

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