*Kidney International, Vol. 57 (2000), pp. 1981–1990*

# ANCA antigens, proteinase 3 and myeloperoxidase, are not expressed in endothelial cells[1]

**William F. Pendergraft, David A. Alcorta, Mårten Segelmark, Jia J. Yang, Robin Tuttle, J. Charles Jennette, Ronald J. Falk, and Gloria A. Preston**

*Division of Nephrology and Hypertension, Department of Medicine, and Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; and Department of Nephrology, Lund University, Lund, Sweden*

**ANCA antigens, proteinase 3 and myeloperoxidase, are not expressed in endothelial cells.**

*Background.* One hypothesis for the pathogenesis of vasculitis associated with antineutrophil cytoplasmic autoantibodies (ANCAs) proposes that ANCAs bind to ANCA antigens, such as proteinase 3 (PR3) or myeloperoxidase (MPO), which are produced by endothelial cells and expressed on their surfaces. There are conflicting reports, however, on whether endothelial cells express the ANCA antigen PR3, and there are no reports on endothelial expression of MPO. The aim of this study was to determine the presence or absence of PR3 and MPO mRNA in both venous and arterial endothelial cells, employing standard reverse transcription-polymerase chain reaction (RT-PCR) techniques and also the quantitative and highly specific method, TaqMan PCR.

*Methods.* RT-PCR (with 3 primer sets) and TaqMan PCR, a method for detecting low copy transcripts, were used to probe for PR3 and MPO transcripts in human endothelial cells from umbilical vein (HUVEC) and artery (HUAEC) and from lung microvascular (HLMVEC). Cells were treated with interferon-$\gamma$ (200 units/mL) or tumor necrosis factor-$\alpha$ (3 or 10 ng/mL) or both.

*Results.* Transcripts for PR3 and/or MPO were not detected in HUVEC, HUAEC, and HLMVEC by standard RT-PCR. Analyses for PR3 protein confirmed that PR3 is not expressed in HUVEC. HUVEC and HUAEC were negative for PR3 and MPO by TaqMan PCR.

*Conclusions.* PR3 and MPO are not expressed in HUVEC, HUAEC, or HLMVEC. Endothelial cell presentation of endogenous PR3 and MPO antigens is not involved in the pathogenesis of ANCA-associated vasculitis. Alternative explanations need to be explored to determine the pathogenic effect of ANCAs.

The endothelium has often been considered a possible target of antineutrophil cytoplasmic autoantibody (ANCA)-mediated effects because of the histologic pattern of necrotizing vascular injury that is the pathologic hallmark of the ANCA-associated vasculitides. A mechanism whereby ANCA would physically interact with endothelial cells is not obvious. The specific ANCA antigens [neutrophil granule proteins, proteinase 3 (PR3), and myeloperoxidase (MPO)] should not be available on the endothelial cell surface for interactions, based on published reports stating that the transcription of these genes is developmentally regulated and restricted to myeloid lineage cells [1, 2]. However, a major controversy has arisen over whether endothelial cells also can synthesize ANCA antigens, especially PR3.

The first report of PR3 expression in endothelial cells was published in 1993 by Mayet et al [3]. In this study, C-ANCA (anti–PR3-autoantibodies) $Fab'_2$ fragments were shown to bind an antigen expressed on IL-1$\alpha$–treated human endothelial cells (HEC). It was proposed that cytokine treatment induced the membrane expression of PR3, based on their observations that peak staining was seen after two hours of incubation, while untreated cells showed only scant staining. Mayet et al later published data showing that C-ANCA were functionally involved in changes in cell signaling events, resulting in the induction of E-selectin and vascular cell adhesion molecule (VCAM) expression [4, 5]. In the case of VCAM expression, however, C-ANCA binding to endothelial cells did not require cytokine induction of PR3 expression. The data spurred a novel theory of how ANCA might mediate injury to the vessel walls, that is, direct antibody–antigen interactions on the endothelial cell surface. Recently, several more reports have described C-ANCA–related effects on endothelial cell function [6–8].

Contrary to the findings of Mayet et al, King et al reported that endothelial cells do not express PR3 [9], and recently, a second group showed that PR3 was not expressed in human endothelial cells from umbilical vein

[1] **See Editorial by Cohen Tervaert, p. 2171.**

**Key words:** PR3, MPO, ANCA, endothelial cells, vasculitis.

Received for publication August 6, 1999
and in revised form October 27, 1999
Accepted for publication December 5, 1999

(HUVECs) [10]. Resolution of the issue of endothelial expression of ANCA antigens is required to move forward toward the elucidation of the pathogenesis of ANCA disease.

The aim of this study was to systematically address the question of PR3 expression by employing standard reverse transcription-polymerase chain reaction (RT-PCR) techniques and also the quantitative and highly specific method TaqMan PCR to detect PR3 and MPO mRNA in endothelial cells. We examined three cell types, including vein endothelial, arterial endothelial, and lung microvascular endothelial cells. The data presented here indicate that PR3 and MPO are not synthesized in endothelial cells.

## METHODS

### Cell culture

Human umbilical vein endothelial cells and human umbilical arterial endothelial cells (HUAEC) from pooled donors were isolated in house from human umbilical cord using collagenase (125 U/mL) in Hank's buffered saline solution (HBSS). Cells were grown on gelatin-coated tissue culture plates in M199 medium, 15% fetal calf serum (FCS; GIBCO, Grand Island, NY, USA), glucose (4.5 g/L), 0.1 mol/L HEPES, L-glutamine (200 mmol/L; GIBCO), Pen/Strep (100 units penicillin, 100 μg streptomycin; GIBCO), heparin (750 U/mL), and endothelial cell growth supplement (ECGS; 50 μg/mL; Becton Dickinson, Mountain View, CA, USA) in 5% $CO_2$. Endothelial cells were characterized by morphology and by positive staining for von Willebrand factor by immunofluorescence (IF). HUVEC (passage 3) and HUAEC (passage 3) from pooled donors, purchased from Clonetics Corporation (San Diego, CA, USA), were cultured on standard tissue culture plates in the endothelial cell basal medium (EBM). Supplements provided were 6 mg bovine brain extract, 5 μg human endothelial growth factor, 0.5 mg hydrocortisone, 50 mg gentamycin, and 2% fetal bovine serum (FBS). HMVEC-Ls (passage 6, human microvascular endothelial cells-lung; Clonetics Corp., San Diego, CA, USA) from pooled donors were cultured on standard tissue culture plates in medium provided (EGM-MV). Supplements provided were identical to HUAEC and HUVEC supplements with the exception of 5% FBS. EA.hy926 cells (a chimeric cell line containing A549 epithelial carcinoma cell and HUVEC components), which express factor VIII-related antigen, were developed and donated by Dr. C.J. Edgell (Department of Pathology, University of North Carolina at Chapel Hill, NC, USA). EA.hy926 cells were cultured in Dulbecco's modified Eagle medium high glucose (DMEM-h) with Pen/Strep (100 units penicillin, 100 μg streptomycin; GIBCO) and 10% FBS. A promyelocytic cell line, HL60, shown to express PR3 and MPO, was used as a positive control. HL60 cells were cultured in RPMI, with Pen/Strep (100 units penicillin, 100 μg streptomycin; GIBCO), and 10% heat-inactivated FBS.

### Cytokine treatment

Human umbilical vein endothelial cells, HMVEC-L, and EA.hy926 cells were treated with 3 ng/mL (30 min) or 10 ng/mL (120 min) recombinant human tumor necrosis factor-α (TNF-α; Sigma Chemical Co., St. Louis, MO, USA). HUAECs and HUVECs were treated with recombinant human interferon-γ (IFN-γ; Genzyme, Cambridge, MA, USA) at 200 U/mL (6 h), TNF-α at 10 ng/mL (6 h), or IFN-γ plus TNF-α.

### RNA isolation and reverse transcription

Cells were harvested at 80% confluency. Media were removed, and RNA STAT-60 (Tel-Test "B," Friendswood, TX, USA) was added to the monolayer without rinsing. Detached cells were collected and included in the RNA preparation. Total RNA was extracted using the supplied protocol. Reverse transcriptase (RT) reactions contained 1 μg total RNA in a 20 μL reaction, containing 200 units of Moloney murine leukemia virus reverse transcriptase, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol (DTT), 40 units RNAse inhibitor and 0.5 μg oligo$(dT)_{15}$ (Promega, Madison, WI, USA). Controls were implemented to detect DNA contamination in the RNA preparations (−RT) by replacing the RT with 1 μL of $H_2O$. Total RNA, diethyl pyrocarbonate-treated $H_2O$, and oligo$(dT)_{15}$ were incubated for two minutes at 70°C and quickly cooled in ice. dNTPs and RT were then added and incubated for 60 minutes at 42°C. The reaction was inactivated at 97°C for five minutes. The volume was increased to 100 μL using diethyl pyrocarbonate (DEPC)-treated $H_2O$.

### Primer sequences

Primers were synthesized by the Oligonucleotide Synthesis Facility (Department of Pathology, University of North Carolina at Chapel Hill): GAPDH forward primer, 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′, GAPDH reverse primer, 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′; PR3 primer set 1 (bp 178 to 547), forward primer, 5′-CTT GAT CCA CCC CAG CTT CGT G-3′, reverse primer, 5′-GCA GAA GAA GGT GAC CAC GGT GAC-3′; PR3 primer set 2 from Mayet et al [3] forward primer, 5′-ATG GCC TCC CTG CAG ATG CGG GGG-3′, reverse primer, 5′-CGG AGG CAC TGA GGT TGG CTG GGC-3′; PR3 primer set 3, forward 5′-CTTCTGCGGAGGCACCTTGATC-3′, reverse 5′-GCGAGGGACGAAAGTGCAAATG-3′; MPO (bp 1654 to 1916) forward primer, 5′-CAC CCT CAT CCA ACC CTT CAT GTT C-3′, MPO reverse

primer, 5′-CAT GTT CAG AGC AGG CAG GTC CAG-3′.

### Polymerase chain reaction parameters

Polymerase chain reaction was done using the Perkin Elmer Thermal Cycler 9600 (Perkin Elmer Corp., Hayward, CA, USA). GAPDH primers were included in each PCR reaction as an internal control. To detect contaminating material in reagents, a sample was run minus cDNA. The reactions consisted of cDNA (0.2 μg), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$, 200 μmol/L dNTP, 20 μmol/L of each primer and two units of AmpliTaq DNA Polymerase (Perkin Elmer). Samples were heated to 95°C for two minutes to denature all cDNA prior to cycling. Parameters used for primer set 1 for PR3 and for MPO primers were 35 cycles of 94°C for 45 seconds, 57°C for 60 seconds, and 72°C for 120 seconds. The Hot Start technique was implemented for MPO primers by heating all samples to 85°C for five minutes prior to its addition to reduce nonspecific priming. Parameters used for PR3 primer set 2 were 30 cycles of 95°C for 60 seconds, 62°C for 60 seconds, and 72°C for 60 seconds. Parameters used for PR3 primer set 3 were 40 cycles of 94°C for 45 seconds, 60°C for 60 seconds, and 72°C for 120 seconds.

### Protein extraction and immunoblotting

Cells were rinsed twice in calcium-free and magnesium-free phosphate-buffered saline (PBS). A volume of nonreducing Laemmli sample buffer (100°C) was added directly to the dish to achieve a final concentration of $3 \times 10^6$ cells/mL. Cell lysates were collected and boiled for five minutes. Aliquots equal to approximately $2 \times 10^5$ cells were subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; 15%) electrophoresis and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, USA) at 100 V for one hour. Blots were blocked in 10% milk-TBST (Tris-buffered saline containing 0.05% Tween 20) for one hour at room temperature. Antibodies used were mouse antihuman PR3 [11], kindly donated by Jorgen Wieslander (Wieslab, AB, Lund, Sweden), goat antihuman $pp90^{rsk}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and antimouse or antigoat secondary conjugated to horseradish peroxidase (Chemicon, Temecula, CA, USA).

### PR3 protein analysis by immunofluorescence

The Department of Pathology (University of North Carolina at Chapel Hill), using standard clinical methods, performed the IF analyses. HUVECs (passage 3) were subcultured onto glass chamber slides (Nalge Nunc International, Rochester, NY, USA) and coated with endothelial cell attachment factor (Sigma Biosciences, St. Louis, MO, USA). Cells were treated with TNF-α (Genzyme Diagnostics, Cambridge, MA, USA) 25 ng/mL for 24 hours and 50 ng/mL for 1 or 24 hours. Cells were washed with PBS, fixed in acetone for 10 minutes, and air dried. Slides were rehydrated for five minutes in PBS. Cells were incubated in primary antibody (diluted in PBS plus 0.2% normal goat serum; Sigma Biosciences) for 30 minutes in a dark, humid container, washed twice in PBS, and then incubated for 20 minutes with a secondary fluorescein-labeled antibody. Primary antibodies included mouse anti-PR3 (5 μg/mL, positive control), a monoclonal antibody developed and donated by Jorgen Wieslander (Wieslab) [11]. This antibody is characterized by sensitive and specific binding to PR3 by indirect IF using normal human neutrophils fixed in acetone. The conditions used for this positive control were those used to detect the presence of PR3 in endothelial cells. Additional primary antibodies included ANCA-positive human serum (1:100) and purified IgG (5 μg/mL), normal human serum (1:100), and purified IgG (5 μg/mL; Pierce, Rockford, IL, USA), rabbit antihuman von Willebrand factor (5 μg/mL; Dako Corporation, Carpinteria, CA, USA), and mouse antihuman VCAM-1 (5 μg/mL; Genzyme Diagnostics). A negative control, containing diluent in place of a primary antibody, was included with each secondary antibody (Jackson Immunoresearch Laboratories, St. Louis, MO, USA) to detect background fluorescence. The mouse anti-PR3 antibody was used as a positive control antibody against PR3. The rabbit antihuman von Willebrand factor was used as a positive control for the endothelial cells, and the mouse anti–VCAM-1 was used to test TNF-α activation at various time points.

### PCR sensitivity assay

PR3cDNA/pAcC4 plasmid was a generous gift from Dr. Joelle E. Gabay (Cornell University Medical College, Division of Infectious Diseases). Full-length PR3 cDNA was digested from the pAcC4 vector and purified using the Qiagen Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). Serial dilutions based on the actual copy number of the cDNA were subjected to RT-PCR using PR3 primer set 3 as described previously in this article. Amplified products were analyzed by agarose gel electrophoresis and were visualized by ethidium bromide staining.

### TaqMan PCR

Quantitative RT and PCR assays were performed in duplicate on a Perkin-Elmer 7700 TaqMan® PCR machine using a Taqman EZ-RT PCR kit (Perkin-Elmer). Standard RT extension, PCR annealing, and amplification temperatures were used as detailed by the Perkin-Elmer TaqMan manual. Primers and TaqMan probes were designed using the Perkin-Elmer computer program Primer Express. The forward primer for human PR3 was TGT CAC CGT GGT CAC CTT CTT (bp 465

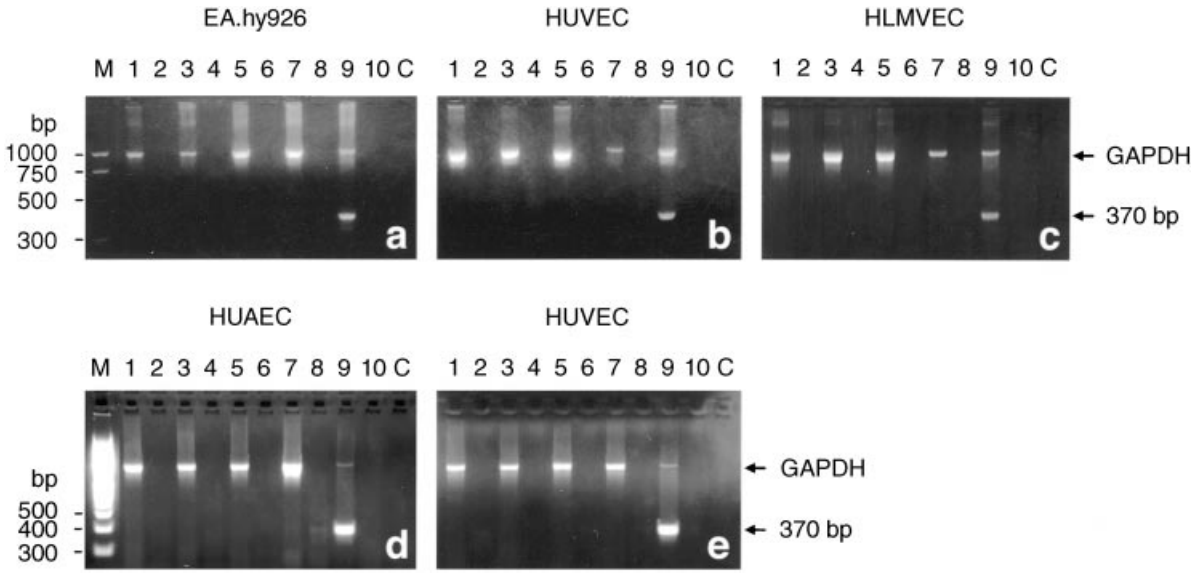



**Fig. 1. Agarose gel analyses of RT-PCR assays performed on endothelial cells to detect proteinase 3 (PR3) message using conditions and primers developed in our laboratory.** HL60 cells positive for PR3 message (*a–e*, lane 9). Untreated human epithelial cells and umbilical vein (HUVEC) and artery HUAEC (a–e, lane 1); tumor necrosis factor-α (TNF-α)–treated cells (a, b, and c, lane 3, 3 ng/30 min; lane 5, 3 ng/2 h; lane 7, 10 ng/2 h); interferon-γ (IFN-γ) 200 U/mL (d and e, lane 5); TNF-α 10 ng/6 h (d and e, lane 3); TNF-α plus IFN-γ (d and e, lane 7). Minus reverse transcriptase controls (−RT; a–e, lanes 2, 4, 6, 8, and 10). GAPDH amplification of approximately 1000 bp can be observed in lanes 1, 3, 5, 7, and 9 (a–e). Abbreviations are: M, molecular weight marker; C, -DNA control; bp, base pairs.

to 485), and the reverse primer was CCC CAG ATC ACG AAG GAG TCT AT (bp 589 to 611). The probe was FAM-TTG CAC TTT CGT CCC TCG CCG-TAMRA (bp 504 to 524). For MPO, primers were CCA GGA AGC CCG GAA GAT (bp 1492 to 1509), CGG AAG GCA TTG GTG AAG A (bp 1641 to 1659) and FAM-TGC CCA CGT ACC GTT CCT ACA ATG ACT C-TAMRA (bp 1590 to 1617) for forward, reverse, and probe oligos, respectively. To increase sensitivity for low-copy number mRNAs, primer concentration was increased to 600 μmol/L and probe increased to 200 μmol/L. Fifty nanograms of total RNA were used per reaction. For the dilution curve calibration, HL60-positive RNA was serially diluted into samples containing 50 ng of HUVEC RNA to maintain the overall quantity of RNA in each sample. Relative quantitation was determined by standard $2^{(-\Delta\Delta Ct)}$ calculations.

## RESULTS

### Analysis of PR3 transcripts

To test for PR3 transcripts in endothelial cells, RT-PCR was used with primers established in our laboratory. cDNA was amplified in the positive-control HL60 cells (Fig. 1), resulting in a band of expected size of 370 bp. PR3 was not detected in the untreated HUVECs and HUAECs or in the TNF-α–treated cells (Fig. 1). IFN-γ or a combination TNF-α + IFN-γ was added to HUVECs and HUAECs, and *PR3* gene activation was evaluated by RT-PCR. Neither untreated endothelial cells showed amplification of PR3. The predicted band of 370 bp was amplified in HL60 cells (Fig. 1). PCR reactions were performed using the primer set and parameters described by Mayet et al [3]. PR3 amplification (expected size of 276 bp) was not detected in EA.hy926, HUVECs, HMVEC-Ls, or HUAECs in both treated and non-treated cells (Fig. 2).

### Analysis of MPO transcripts

Polymerase chain reaction was performed to determine whether MPO is expressed in HUAECs and/or HUVECs. The predicted 262 bp product was observed in the HL60-positive control cell line (Fig. 3). MPO transcripts were not detected in the nontreated cells or in the treated cells (Fig. 3). EA.hy926 cells and HMVEC-L were also negative for MPO (data not shown). The data indicate that MPO is not expressed in endothelial cells.

### Analysis of PR3 protein

Proteinase 3 protein was not detected in HUVEC by IF (Table 1), using two anti-PR3 antibodies. PR3 reactivity was negative in 17 out of 18 reactions, although as shown, in this particular experiment, a slight positive was recorded in one sample. Repeat experiments showed this to be a false positive because of the high background. PR3 protein was not detected by Western analysis (Fig. 4) of untreated HUVECs (lane 2) or after TNF-α treatment (lanes 3 through 6). Purified PR3 protein (lane 1)

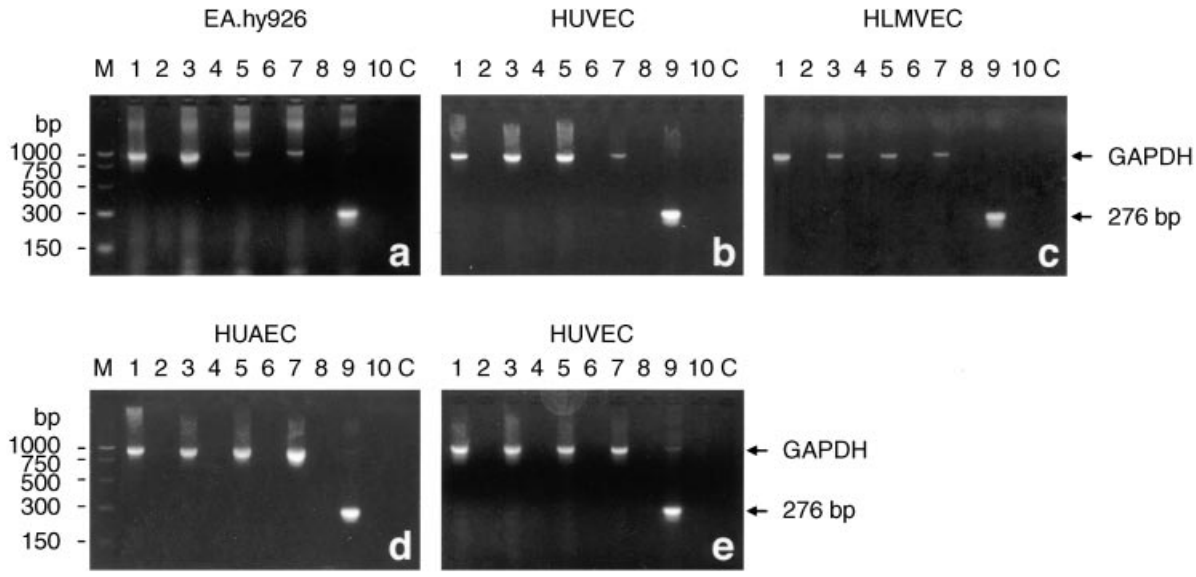



**Fig. 2. Agarose gel analyzes of RT-PCR assays performed on endothelial cells to detect PR3 message using conditions and primers developed by Mayet et al.** Untreated EA.hy926 cells (a, lane l), HUVEC (b and e, lane 1), human lung microvascular endothelial cells (HLMVEC; c, lane 1); treated with TNF-α 3 ng/mL for 30 minutes (a, b, and c, lane 3); TNF-α 3 ng/mL for two hours (a, b, and c, lane 5); TNF-α 10 ng/mL for two hours (a, b, and c, lane 7). HUAECs (d) and HUVECs (e) treated with TNF-α 10 ng/mL for six hours (d and e, lane 3), IFN-γ 200 U/mL for six hours (d and e, lane 5), and TNF-α 10 ng/mL plus IFN-γ 200 U/mL for six hours (d and e, lane 7). The HL60 cells were used as a positive control for PR3 message (a–e, lane 9). −RT controls were implemented for each sample (a–e, lanes 2, 4, 6, 8, and 10). M, molecular weight marker; C, -DNA control; bp, base pairs.

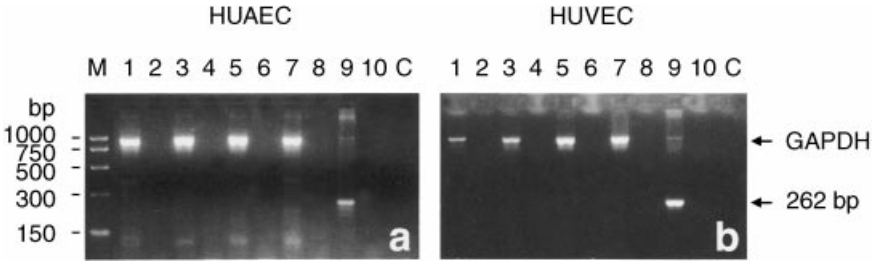



**Fig. 3. Agarose gel analyses of RT-PCR assays performed on endothelial cells to detect myeloperoxidase (MPO).** HL60-positive control for MPO expression (a and b, lane 9). MPO transcripts were not detected in the nontreated cells (a and b, lane 1) or in the treated cells (a and b, lane 3, TNF-α 10 ng/6 h; lane 5, IFN-γ 200U/mL; lane 7, IFN-γ + TNF-α 6 h). −RT controls were implemented for each sample (lanes 2, 4, 6, 8, and 10, a and b). Abbreviations are: M, molecular weight marker; C, -DNA control; bp, base pairs.

ran at a slightly lower mobility than PR3 in HL60 cells. The nitrocellulose was reprobed with anti-pp90$^{rsk}$ to confirm that similar amounts of protein were loaded.

### PCR sensitivity analysis

If PR3 and/or MPO mRNA were present in HUVECs, it is highly unlikely that we would not have detected it, considering that PCR technology is sensitive enough to detect 10 molecules or less of mRNA per cell [12]. Since 200 ng of total RNA (roughly 20,000 cells) were used for each RT reaction in this study, there would have been sufficient material to amplify any existing low levels of PR3 and/or MPO message. Cytokine-induced transcripts should have been present, since the half-life of MPO and PR3 transcripts is approximately 3.5 hours [13]. However, to test the level of detection of PCR in our system, serial dilutions of the purified full-length PR3 cDNA were prepared, and PCR was performed using a third set of PR3 primers (Fig. 5). Based on these data, if there were 100 or more copies (lane 4) of PR3 present in the total reaction, they should have been detected.

### Determination of potential PCR inhibitory factors in HUVEC and HUAEC preparations

One explanation for the lack of PR3 amplification in HUVECs and HUAECs could be that the PCR is inhibited by some component of the HUVEC or HUAEC RNA samples. To test this hypothesis, HL60 cDNA (at varying concentrations) was spiked into HUVEC or HUAEC cDNA. Standard RT-PCR, using primer set 3, was performed. There was a hint that there might be inhibition of PR3 amplification in HUVECs; therefore, we repeated the experiment using the more quantitative TaqMan PCR,

**Table 1.** Analysis of PR3 protein expression by immunofluorescence

| Passage | [TNF] | Hours | Primary antibodies | Results |
|---|---|---|---|---|
| 3 | — | — | Mab anti-PR3 | negative |
| 3 | — | — | hu anti-PR3 | negative |
| 4 | — | — | Mab anti-PR3 | negative |
| 4 | — | — | hu anti-PR3 | negative |
| 5 | — | — | hu anti-PR3 | negative |
| 3 | 20 ng/mL | 1 hour | Mab anti-PR3 | 1+cyto |
| 3 | 20 ng/mL | 1 hour | hu anti-PR3 | negative |
| 3 | 20 ng/mL | 2 hours | Mab anti-PR3 | negative |
| 3 | 20 ng/mL | 2 hours | hu anti-PR3 | negative |
| 3 | 20 ng/mL | 3 hours | Mab anti-PR3 | negative |
| 3 | 20 ng/mL | 3 hours | hu anti-PR3 | negative |
| 4 | 25 ng/mL | 24 hours | Mab anti-PR3 | negative |
| 4 | 25 ng/mL | 24 hours | hu anti-PR3 | negative |
| 4 | 50 ng/mL | 1 hour | Mab anti-PR3 | negative |
| 4 | 50 ng/mL | 1 hour | hu anti-PR3 | negative |
| 4 | 50 ng/mL | 24 hours | Mab anti-PR3 | negative |
| 4 | 50 ng/mL | 24 hours | hu anti-PR3 | negative |
| 5 | 50 ng/mL | 1 hour | hu anti-PR3 | negative |

Abbreviations are: PR3, proteinase 3; [TNF], tumor necrosis factor concentration; Mab, monoclonal antibody; hu, human.

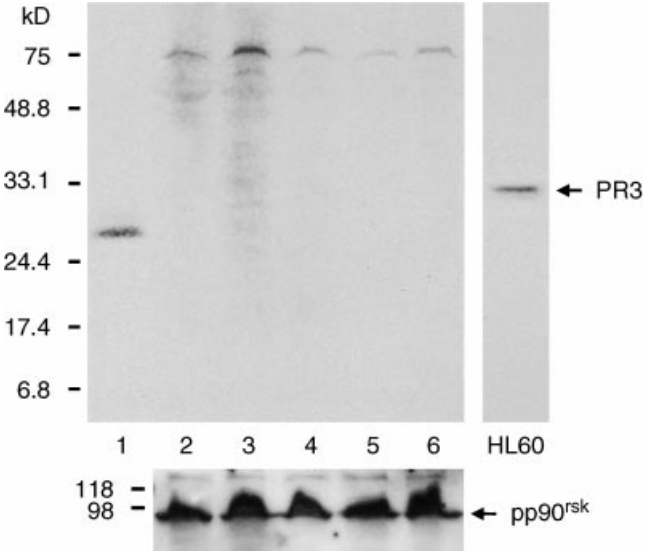



**Fig. 4. Western blot probed with MαPR3 antibody (Wieslab, AB, Lund, Sweden).** Purified PR3 (lane 1; kindly donated by Jorgen Wieslander); HUVEC untreated (lane 2); TNF-α 30 minutes (lane 3), 1 hour (lane 4), 3 hours (lane 5), and 4 hours (lane 6). The nitrocellulose was reprobed with anti-pp90$^{rsk}$ to confirm protein loading.

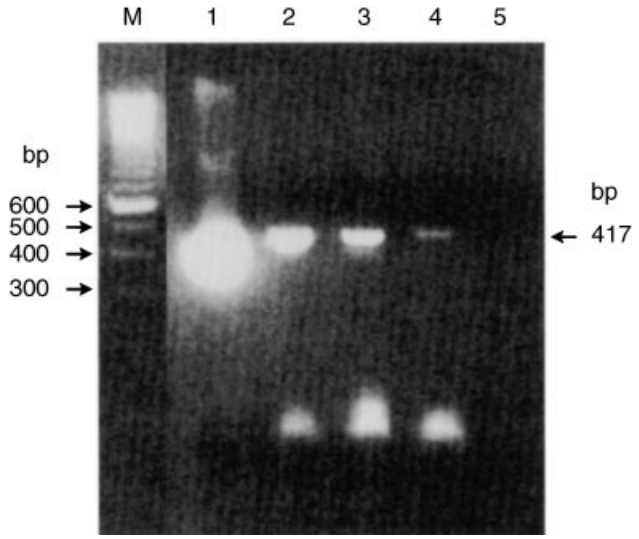



**Fig. 5. PR3 plasmid PCR sensitivity assay.** Purified PR3 cDNA was quantitated and diluted based on copy number. Diluted samples were then subjected to PCR. Lanes 1, 2, 3, 4, and 5 represent PCR amplification of 10,000, 1,000, 500, 100, and 5 copies starting material, respectively. Abbreviation M is molecular weight marker.

and we saw no inhibitory activity in either HUAECs or HUVECs. We conclude that HL60 PR3 was amplified in a HUAEC background (Fig. 6, lanes 3 and 4) and in a HUVEC background (Fig. 6, lanes 7 and 8).

### Specific and quantitative TaqMan PCR assessment of PR3 and MPO mRNA

TaqMan is a one-tube RT-PCR technique [14]. The assay is based on the 5′ nuclease activity of the Thermus flavus polymerase and a fluorogenic probe, which generates fluorescence when it is cleaved. The fluorogenic probe is an oligonucleotide designed to bind between the two PCR primers to the target cDNA, and is labeled with a reporter and a quencher dye. In the intact probe, the quencher dye suppresses the fluorescence of the reporter dye. During the polymerase extension steps, the Thermus flavus exonuclease activity cleaves the hybridized probe, resulting in the separation of the quenching dye from the fluorescent dye. When stimulated, the fluorescence intensity is proportional to the amount of PCR product synthesized. Fluorescence is monitored every cycle for both the gene of interest and GAPDH. The threshold cycle (CT value) is the cycle number in which the PCR amplification as shown by increasing fluorescence is linear at a threshold above background. After completion of amplification, the CT values of the samples are normalized based on the CT values for the GAPDH, which show the relative amounts of input RNA used for each sample. Theoretically, TaqMan has the ability to detect a single transcript per sample. A typical TaqMan data printout is in Figure 7, which shows a standard dilution curve of HL60 RNA, spiked into HUVEC RNA (to attain 50 ng total RNA starting material) probed for PR3 transcripts. Fluorescence on the *y* axis is plotted against PCR cycle number on the *x* axis. The log increase in fluorescence intensity is proportional to transcript copy number. PR3 mRNA in 50 ng of HL60 RNA produced a CT value of 17. A tenfold dilution of HL60 RNA starting material pushed the detection level up to 21 cycles, 100-fold up to 25 cycles, 1000-fold to 28 cycles, 3300-fold to 30 cycles, 10,000-fold to 32 cycles, and

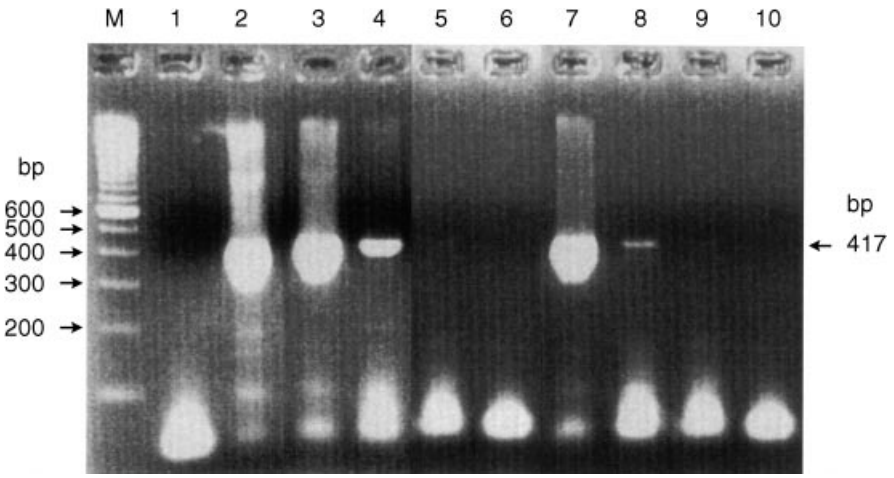



**Fig. 6. PR3 RT-PCR assay for factors that inhibit amplification in endothelial cell preparations.** TNF-α 10 ng/mL treated HUAECs (lane 3, 1:100 HL60 cDNA; lane 4, 1:10,000 HL60 cDNA) and HUVECs (lane 7, 1:100 HL60 cDNA; lane 8 1:10,000 HL60 cDNA). TNF-α 10 ng/mL treated HUAECs (lane 6) and HUVECs (lane 10) without HL60 cDNA. HL60-positive control (lane 2). Abbreviation M is molecular weight marker.

33,000-fold to 35 cycles (100,000-fold dilution showed no amplification after 40 cycles; data not shown). Because the HL60 RNA was added to HUVEC RNA and amplification of PR3 was observed, it can be concluded that in TaqMan PCR PR3 amplification was not inhibited, as was shown for standard PCR, by some unknown agent in the HUVEC preparations. Although TaqMan does not require post-PCR sample agarose gel analysis, the samples generated were evaluated by ethidium bromide staining. Figure 7 (lanes 1 through 6) shows the HL60 dilution curve.

To determine whether HUVECs or HUAECs express PR3, the TaqMan reactions were performed. Of the 36 HUVEC and HUAEC samples tested (from 4 individual experiments), there was no amplification detected of PR3 after 40 cycles. CT values for HUVECs and HUAECs were 40 and were off the scale of the graph (Fig. 7). This means that if PR3 message is present in HUVECs or HUAECs, then the level of PR3 mRNA is 120,000-fold less than that in HL60 cells. Similar experiments and results were obtained for MPO expression in HUVECs and HUAECs relative to HL60 expression (Fig. 8).

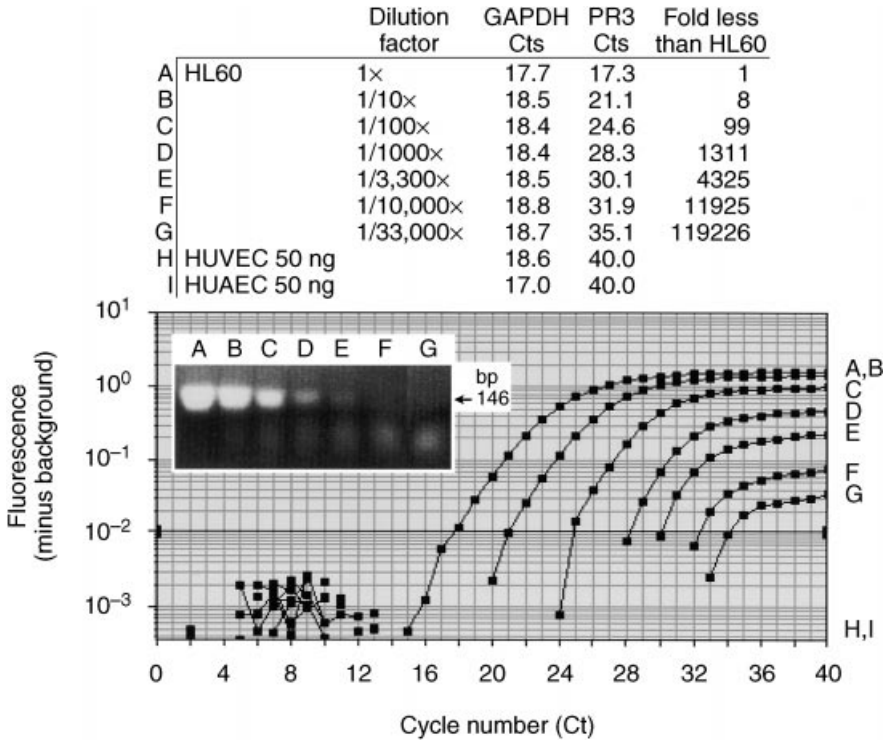
## DISCUSSION

One hypothetical mechanism of ANCA-associated vasculitis is through direct interactions between the vascular endothelial cells and the autoantibodies. This interaction is proposed to be facilitated through endothelial expression and presentation of the PR3 antigen, the same antigen expressed by neutrophils. The studies described here use conventional RT-PCR and TaqMan PCR to address the hotly debated question of whether endothelial cells express PR3. Our data support the conclusion that ANCA antigens PR3 and MPO are not expressed by endothelial cells. These data do not completely rule out the possibility that endothelial cells from other organs or from other individuals treated with other agents could have some transcription of the *PR3* gene. However, we emphasize that our studies included the use of umbilical vein endothelial cells harvested on site from multiple donors, as well as commercial preparations of umbilical vein endothelial cells and endothelial cells from sites not previously examined, including arterial and lung microvascular cells. Our analyses included evaluations of cells exposed to two different cytokines harvested at multiple times after treatment. Therefore, the findings in the present study make it highly unlikely that PR3 production by an endothelial cell is a common and abundant feature, and that such a production would be responsible for the major pathogenic events in ANCA-associated systemic vasculitis.

The demonstration that the PR3 message could be amplified in HUVEC RNA spiked with small amounts of PR3-positive HL60 RNA rules out the possibility that the RT-PCR reactions were blocked in some way in the HUVEC preparations.

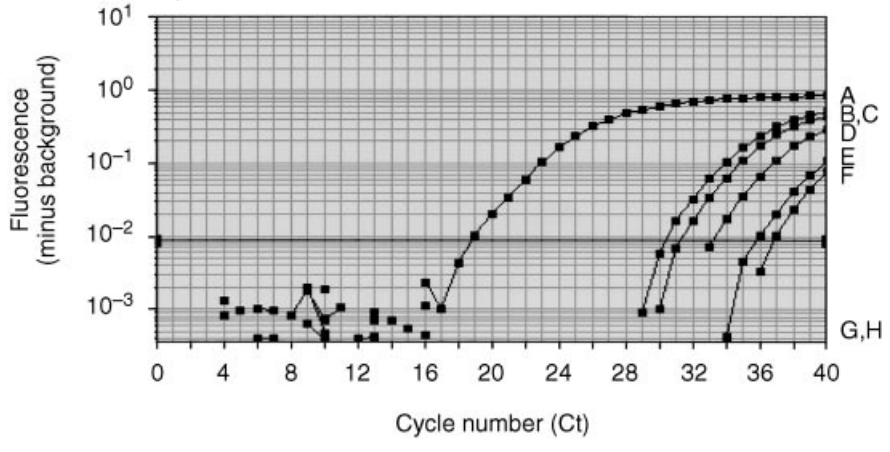
To our knowledge, this is the first report of the application of TaqMan PCR to address the issue of endothelial cells expressing PR3. The high sensitivity, simplicity, and reproducibility of the real-time RNA quantitation, combined with its wide dynamic range, make this method especially suitable. TaqMan PCR is a widely acclaimed method that is now being applied to clinical issues, such as assessment of hepatitis C virus load in serum samples [15], detection of gene amplification in cancers [16–18], detection of mycobacterium tuberculosis DNA in sputum [19], and detection of Salmonella in raw meat [20]. We believe that the use of TaqMan PCR in our investigations has provided the accuracy and reliability necessary to be confident in our results.

P-ANCA (anti-MPO autoantibody) are associated with vasculitis as well; therefore, endothelial cell expression of MPO would be equally important in the pathogenesis of ANCA-mediated disease. The present study




| | | Dilution factor | GAPDH Cts | PR3 Cts | Fold less than HL60 |
|---|---|---|---|---|---|
| A | HL60 | 1× | 17.7 | 17.3 | 1 |
| B | | 1/10× | 18.5 | 21.1 | 8 |
| C | | 1/100× | 18.4 | 24.6 | 99 |
| D | | 1/1000× | 18.4 | 28.3 | 1311 |
| E | | 1/3,300× | 18.5 | 30.1 | 4325 |
| F | | 1/10,000× | 18.8 | 31.9 | 11925 |
| G | | 1/33,000× | 18.7 | 35.1 | 119226 |
| H | HUVEC 50 ng | | 18.6 | 40.0 | |
| I | HUAEC 50 ng | | 17.0 | 40.0 | |

**Fig. 7. TaqMan RT-PCR for PR3.** HUVEC (H) and HUAEC (I) samples were negative for PR3 showing Ct values of 40, off the scale of the graph. Serial dilutions of HL60 RNA were added to HUVEC RNA (*A–G*) to establish a standard curve.




| | | Dilution factor | GAPDH Cts | MPO Cts | Fold less than HL60 |
|---|---|---|---|---|---|
| A | HL60 | 1× | 17.0 | 19.1 | 1 |
| B | | 1/1000× | 17.4 | 30.5 | 2069 |
| C | | 1/3300× | 17.3 | 31.8 | 5773 |
| D | | 1/10000× | 17.1 | 33.7 | 25620 |
| E | | 1/33000× | 17.1 | 36.0 | 120194 |
| F | | 1/100000× | 17.1 | 36.7 | 189916 |
| G | HUVEC T24 | | 17.5 | 40.0 | |
| H | HUAEC T24 | | 16.7 | 40.0 | |

**Fig. 8. TaqMan RT-PCR for MPO.** HUVEC (H) and HUAEC (I) samples were negative for PR3 showing Ct values of 40, off the scale of the graph. Serial dilutions of HL60 RNA were added to HUVEC RNA (*A–G*) to establish a standard curve.

represents the first published data testing for MPO mRNA in endothelial cells. We predicted that if the PR3 gene was transcribed in endothelial cells, and then the *MPO* gene would be also, since de novo synthesis of neutrophil primary granule enzymes is generally coregulated [21–23]. Also, the fact that PR3 and MPO functions are interrelated would imply that coexpression would be biologically favorable [24]. PR3 proteolytic activity

requires MPO-derived products to oxidize and inactivate the PR3 inhibitor α1-antitrypsin. MPO message could not be detected, lending support to the conclusion the PR3 is not expressed in endothelial cells.

This finding is not surprising in light of the published data concerning the requirements for transcription of the *PR3* gene. Two transcriptional control elements are essential for the expression of PR3: PU.1 and CG [1]. The PU.1 (Spi-1) transcription factor is itself predominantly expressed in myeloid and B cells [2, 25]. It is a member of the ETS oncogene family of transcriptional activators critical for development of both these lineages [26], as demonstrated in PU.1−/− mice that lack both lymphoid and myeloid cells [27]. Thus, PU.1 expression is proposed to be a genetic determinant of lineage commitment in blood cell development. PU.1 activates the transcription of lineage-specific proteins, such as MPO [28–30], elastase [27], granulocyte colony-stimulating factor receptor [31], and a variety of other hematopoietic-specific genes [32–38]. In light of the specific factors required for PR3 expression described above, it becomes increasingly apparent that in order to induce transcription of PR3 in endothelial cells, PU.1 transcription factor would be required. However, there are published data showing that PU.1 is not expressed in endothelial cells, osteocytes, or other nonhematopoietic tissues [25, 39].

The potential role of the autoantibodies in the pathogenic process leading to vasculitis remains to be determined. In vitro studies strongly support the theory that ANCA–neutrophil interactions are primarily involved [40–44]. The central questions are whether PMNs in patients are primed, thus resulting in PR3 plasma membrane expression, or whether PMNs in these individuals just normally express PR3/MPO on the cell surface. A recent report has introduced new data showing that a subset of neutrophils from any one individual expresses PR3 molecules on the surface and that the proportion of neutrophils presenting PR3 is highly stable. Plus, this proportion may be genetically controlled, without any relationship to state of activation [45]. Witko-Sarsat et al define this phenomenon as a new polymorphism in the healthy population subdivided into three discrete groups: <20% mPR3+ cells as low, 47% as intermediate, and 71.5% as high. The implication would be that individuals with high expression of PR3 would be at a greater risk for ANCA-mediated disease. Indeed, the authors found that the frequency of the mPR3 high phenotype was significantly increased in patients with ANCA-associated vasculitis. This frequency did not change with disease activity, relapses, or therapy, and did not reflect in vivo cell activation. Determination of the distribution of PR3 expression in other population groups is needed to support these findings, but until then, the controversy of whether cell priming is required for PR3 cell surface expression remains.

Antineutrophil cytoplasmic autoantibody interactions with antigens planted on bystander cells, such as endothelial cells, may be a contributing factor to vascular damage [46]. Alternatively, a recent study by De Bandt et al described antigens on endothelial cells that were recognized by C-ANCA, none of which were PR3 [10]. C-ANCA were shown to complex with surface proteins of 82, 110, and 125 kD in size. If these data are substantiated, they may provide one explanation for reported observations of ANCA-mediated endothelial effects.

In summary, our study indicates that endothelial cells do not express ANCA antigens, PR3 and MPO, and confirms the report by King et al [9].

*Reprint requests to Gloria Preston, Ph.D., University of North Carolina at Chapel Hill, Division of Nephrology and Hypertension, CB# 7155, 346 MacNider Building, Chapel Hill, North Carolina 27599, USA. E-mail: Gloria_Preston@med.unc.edu*

## REFERENCES

1. Sturrock A, Franklin KF, Hoidal JR: Human proteinase-3 expression is regulated by PU.1 in conjunction with a cytidine-rich element. *J Biol Chem* 271:32392–32402, 1996
2. Chen HM, Zhang P, Voso MT, Hohaus S, Gonzalez DA, Glass CK, Zhang DE, Tenen DG: Neutrophils and monocytes express high levels of PU-1 (Spi-1) but not Spi-B. *Blood* 85:2918–2928, 1995
3. Mayet WJ, Csernok E, Szymkowiak C, Gross WL, Meyer ZUM, Buschenfelde KH: Human endothelial cells express proteinase-3, the target antigen of anticytoplasmic antibodies in Wegener's granulomatosis. *Blood* 82:1221–1229, 1993
4. Mayet WJ, Meyer ZUM, Buschenfelde KH: Antibodies to proteinase 3 increase adhesion of neutrophils to human endothelial cells. *Clin Exp Immunol* 94:440–446, 1993
5. Mayet WJ, Schwarting A, Orth T, Duchmann R, Meyer ZUM, Buschenfelde KH: Antibodies to proteinase 3 mediate expression of vascular cell adhesion molecule-1 (VCAM-1). *Clin Exp Immunol* 103:259–267, 1996
6. Sibelius U, Hattar K, Schenkel A, Noll T, Csernok E, Gross WL, Mayet WJ, Piper HM, Seeger W, Grimminger F: Wegener's granulomatosis: Anti-proteinase 3 antibodies are potent inductors of human endothelial cell signaling and leakage response. *J Exp Med* 187:497–503, 1998
7. Mayet WJ, Schwarting A, Orth T, Sibelius U, Hattar K, Meyer ZUM, Buschenfelde KH: Signal transduction pathways of membrane expression of proteinase 3 (PR-3) in human endothelial cells. *Eur J Clin Invest* 27:893–899, 1997
8. Johnson PA, Alexander HD, Mcmillan SA, Maxwell AP: Upregulation of the endothelial cell adhesion molecule intercellular adhesion molecule-1 (ICAM-1) by autoantibodies in autoimmune vasculitis. *Clin Exp Immunol* 108:234–242, 1997
9. King WJ, Adu D, Daha MR, Brooks CJ, Radford AA, Savage COS: Endothelial cells and renal epithelial cells do not express the Wegener's autoantigen, proteinase 3. *Clin Exp Immunol* 102:98–105, 1995
10. De Bandt M, Meyer O, Dacosta L, Elbim C, Pasquier C: Anti-proteinase-3 (PR3) antibodies (C-ANCA) recognize various targets on the human umbilical vein endothelial cell (HUVEC) membrane. *Clin Exp Immunol* 115:362–368, 1999
11. Sommarin Y, Rasmussen N, Wieslander J: Characterization of monoclonal antibodies to proteinase-3 and application in the study of epitopes for classical anti-neutrophil cytoplasm antibodies. *Exp Nephrol* 3:249–256, 1995
12. Kawasaki ES, Clark SS, Coyne MY, Smith SD, Champlin R, Witte ON, McCormick FP: Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA

sequences amplified in vitro. *Proc Natl Acad Sci USA* 85:5698–5702, 1988
13. Chen T, Meier R, Ziemiecki A, Fey MF, Tobler A: Myeloblastin/proteinase 3 belongs to the set of negatively regulated primary response genes expressed during in vitro myeloid differentiation. *Biochem Biophys Res Commun* 200:1130–1135, 1994
14. Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H: One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. *J Virol Methods* 77:37–46, 1999
15. Martell M, Gomez J, Esteban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J: High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* 37:327–332, 1999
16. Bieche I, Olivi M, Champeme MH, Vidaud D, Lidereau R, Vidaud M: Novel approach to quantitative polymerase chain reaction using real-time detection: Application to the detection of gene amplification in breast cancer. *Int J Cancer* 78:661–666, 1998
17. Laurendeau I, Bahuau M, Vodovar N, Larramendy C, Olivi M, Bieche I, Vidaud M, Vidaud D: TaqMan PCR-based gene dosage assay for predictive testing in individuals from a cancer family with INK4 locus haploinsufficiency. *Clin Chem* 45:982–986, 1999
18. Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, Vidaud M: Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. *Cancer Res* 59:2759–2765, 1999
19. Desjardin LE, Perkins MD, Wolski K, Haun S, Teixeira L, Chen Y, Johnson JL, Ellner JJ, Dietze R, Bates J, Donald CM, Eisenach KD: Measurement of sputum mycobacterium tuberculosis messenger RNA as a surrogate for response to chemotherapy. *Am J Respir Crit Care Med* 160:203–210, 1999
20. Kimura B, Kawasaki S, Fujii T, Kusunoki J, Itoh T, Flood SJ: Evaluation of TaqMan PCR assay for detecting Salmonella in raw meat and shrimp. *J Food Prot* 62:329–335, 1999
21. Berliner N, Hsing A, Graubert T, Sigurdsson F, Zain M, Bruno E, Hoffman R: Granulocyte colony-stimulating factor induction of normal human bone marrow progenitors results in neutrophil-specific gene expression. *Blood* 85:799–803, 1995
22. Anderson KL, Smith KA, Pio F, Torbett BE, Maki RA: Neutrophils deficient in PU.1 do not terminally differentiate or become functionally competent. *Blood* 92:1576–1585, 1998
23. Grisolano JL, Ley TJ: Regulation of azurophil granule-associated serine protease genes during myelopoiesis. *Curr Opin Hematol* 3:55–62, 1996
24. Weiss SJ: Tissue destruction by neutrophils. *N Engl J Med* 320:365–376, 1989
25. Hromas R, Orazi A, Neiman Rs Maki R, Van Beveran C, Moore J, Klemsz M: Hematopoietic lineage- and stage-restricted expression of the ETS oncogene family member PU.1. *Blood* 82:2998–3004, 1993
26. Chen H, Ahang P, Radomska HS, Hetherington CJ, Zhang DE, Tenen DG: Octamer binding factors and their coactivator can activate the murine PU.1 (spi-1) promoter. *J Biol Chem* 271:15743–15752, 1996
27. Scott EW, Simon MC, Anastasi J, Singh H: Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265:1573–1577, 1994
28. Zhao WG, Regmi A, Austin ED, Braun JE, Racine M, Austin GE: Cis-elements in the promoter region of the human myeloperoxidase (MPO) gene. *Leukemia* 10:1089–1103, 1996
29. Nuchprayoon I, Meyers S, Scott LM, Suzow J, Hiebert S, Friedman AD: PEBP2/CBF, the murine homolog of the human AML1 and PEBP2 Beta/CBF beta proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. *Mol Cell Biol* 14:5558–5568, 1994
30. Piedrafita FJ, Molander RB, Vansant G, Orlova EA, Pfahl M, Reynolds WF: An alu element in the myeloperoxidase promoter contains a composite SP1-thryoid hormone-retinoic acid response element. *J Biol Chem* 271:14412–14420, 1996
31. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG: PU.1 (Spi-1) and c/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 88:1234–1247, 1996
32. Feinman R, Qiu WO, Pearse RN, Nikolajczyk BS, Sen R, Shefffery M, Ravetch JV: PU.1 and an HLH family member contribute to the myeloid-specific transcription of the Fc gamma RIIIA promoter. *EMBO J* 13:3852–3860, 1994
33. Schwarqenbach H, Newell JW, Matthias P: Involvement of the Ets family factor PU.1 in the activation of immunoglobulin promoters. *J Biol Chem* 270:898–907, 1995
34. Henkelgab MA: PU.1 and Gata: Components of a mast cell-specific interleukin 4 intronic enhancer. *Proc Natl Acad Sci USA* 91:7737–7741, 1994
35. Moulton KS, Semple K, Wu H, Glass CK: Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol Cell Biol* 14:4408–4418, 1994
36. Singh H: Genetic analysis of transcription factors implicated in B lymphocyte development. *Immunol Res* 13:280–290, 1994
37. Hitomi Y, Yamada T, Oikawa T: Extinction of expression of the PU.1/Sfpi-1 putative oncogene encoding a B-cell- and macrophage-specific transcription factor in somatic cell hybrids. *Cancer Res* 53:5759–5765, 1993
38. Eisenbeis CF, Singh H, Storb U: PU.1 is a component of a multiprotein complex which binds an essential site in the murine immunoglobulin lambda 2-4 enhancer. *Mol Cell Biol* 13:6452–6461, 1993
39. Galson DL, Hensold JO, Bishop TR, Schalling M, D'Andrea AD, Jones C, Auron PE, Houseman DE: Mouse beta-globin DNA-binding protein B1 is identical to proto-oncogene, the transcription factor Spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis. *Mol Cell Biol* 13:2929–2941, 1993
40. 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
41. Kettritz R, Jennette JC, Falk RJ: Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol* 8:386–394, 1997
42. Brooks CJ, King WJ, Radford DJ, Adu D, McGrath M, Savage CO: IL-1 beta production by human polymorphonuclear leukocytes stimulated by anti-neutrophil cytoplasmic autoantibodies: Relevance to systemic vasculitis. *Clin Exp Immunol* 106:273–279, 1996
43. Lai KN, Lockwood CM: The effect of anti-neutrophil cytoplasm autoantibodies on the signal transduction of human neutrophils. *Clin Exp Immunol* 85:396–401, 1991
44. Grimminger F, Hattar K, Papavassilis C, Temmesfeld B, Csernok E, Gross WL, Seeger W, Sibelius U: Neutrophil activation by anti-proteinase 3 antibodies in Wegener's granulomatosis: Role of exogenous arachidonic acid and leukotriene B4 generation. *J Exp Med* 184:1567–1572, 1996
45. Witko-Sarsat V, Lesavre P, Lopez S, Bessou G, Hieblot C, Prum B, Noel LH, Guillevin L, Ravaud P, Sermet-Gaudelus I, Timsit J, Grunfeld J, Halbwachs-Mecarelli L: A large subset of neutrophils expressing membrane proteinase 3 is a risk factor for vasculitis and rheumatoid arthritis. *J Am Soc Nephrol* 10:1224–1233, 1999
46. Savage COS, 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