

Wegener's Granulomatosis: Anti-proteinase 3 Antibodies Are Potent Inductors of Human Endothelial Cell Signaling and Leakage Response

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Summary

Anti-neutrophil cytoplasmic antibodies (ANCA) targeting proteinase 3 (PR3) have a high specificity for Wegener's granulomatosis (WG), and their role in activating leukocytes is well appreciated. In this study, we investigated the influence of PR3-ANCA and murine monoclonal antibodies on human umbilical vascular endothelial cells (HUVECs). Priming of HUVECs with tumor necrosis factor α induced endothelial upregulation of PR3 message and surface expression of this antigen, as measured by Cyto-ELISA, with a maximum occurrence after 2 h. Primed cells responded to low concentrations of both antibodies (25 ng–2.5 μ g/ml), but not to control immunoglobulins, with pronounced, dose-dependent phosphoinositide hydrolysis, as assessed by accumulation of inositol phosphates. The signaling response peaked after 20 min, in parallel with the appearance of marked prostacyclin and platelet-activating factor synthesis. The F(ab)₂ fragment of ANCA was equally potent as ANCA itself. Disruption of the endothelial F-actin content by botulinum C2 toxin to avoid antigen-antibody internalization did not affect the response. In addition to the metabolic events, anti-PR3 challenge, in the absence of plasma components, provoked delayed, dose-dependent increase in transendothelial protein leakage. We conclude that anti-PR3 antibodies are potent inductors of the preformed phosphoinositide hydrolysis-related signal transduction pathway in human endothelial cells. Associated metabolic events and the loss of endothelial barrier properties suggest that anti-PR3-induced activation of endothelial cells may contribute to the pathogenetic sequelae of autoimmune vasculitis characterizing WG.

The diagnosis of Wegener's granulomatosis (WG),¹ a systemic vasculitis that may affect several organs and has poor prognosis in full-blown cases, has largely profited from the discovery of anti-neutrophil cytoplasmic antibodies (ANCA; references 1, 2). Based on immunofluorescence patterns, the cytoplasmic (classic) ANCA (c-ANCA), targeting proteinase 3 (PR3) contained in azurophilic granules

(3, 4), and the perinuclear ANCA, brought about by anti-myeloperoxidase antibodies (5, 6), are distinguished. The presence of c-ANCA has a nearly 95% specificity for WG, and the titer correlates well with disease activity (7, 8). Additional autoantigenic ANCA targets have recently been identified (9).

Besides being a seromarker of WG, there is now good evidence for a pathogenetic role of c-ANCA. When being primed with cytokines, as occurs in episodes of infection or inflammation, neutrophils express PR3 on their surface, which thus becomes accessible to autoantibody binding (10–13). In vitro studies demonstrated that such binding provokes respiratory burst and degranulation (6, 12, 14, 15), and these inflammatory events are largely amplified in the presence of free arachidonic acid assumed to arise in the microenvironmental milieu of an inflammatory focus (16).

¹Abbreviations used in this paper: ANCA, anti-neutrophil cytoplasmic antibodies; c-ANCA, cytoplasmic ANCA; HUVEC, human umbilical cord endothelial cell; IP, inositol phosphate; IPx, IP1, IP2, and IP3; MoAb-PR3, mAb to proteinase 3; PAF, platelet-activating factor; PR3, proteinase 3; WG, Wegener's granulomatosis.

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These findings suggest that endothelial cell stimulation and injury, a hallmark of WG's granulomatosis, may be a consequence of antibody-related neutrophil activation, as reproduced in vitro and in experimental studies (17–19). Recently, however, evidence was presented that PR3, the target antigen of c-ANCA, may also be present on the surface of endothelial cells under conditions of cytokine priming (20). Moreover, the data clearly supported the notion that the endothelial PR3 surface expression was not due to binding of exogenous PR3, but to upregulation of endogenous PR3 synthesis and its transfer to the endothelial cell surface. Admixture of anti-PR3 antibodies to such cells caused enhanced expression of the adhesion molecules endothelial leukocyte adhesion molecule 1 (ELAM-1) (21) and vascular cell adhesion molecule 1 (VCAM-1) (22), which might again favor interaction with leukocytes. Using c-ANCA-positive serum from WG patients and an mAb manufactured against human PR3 (MoAB-PR3), we now investigated anti-PR3-related alterations in human endothelial cell biology in more detail. Interestingly, pronounced activation of the phosphoinositide hydrolysis-related signal transduction pathway was noted, alongside with induction of lipid mediator generation. In addition, barrier properties of the endothelial cell monolayer, assessed in the absence of plasma components and neutrophils, were progressively lost. These data suggest that hitherto not recognized direct endothelial cell activation by c-ANCA may contribute to the development of vascular injury in WG.

Materials and Methods

Preparation of Human Umbilical Vascular Endothelial Cells. Isolation and culturing were performed as previously described (23, 24). Cells of 10 donors were pooled to exclude the influence of blood group antigens. Morphology was confirmed by phase-contrast light microscopy (cobblestone monolayer appearance), and purity was tested with antibodies to von Willebrand's factor.

Antibody Preparation. Human MoAb-PR3s were prepared as previously described (11); controls were performed with murine mAb IgG, isotype control (Dianova, Hamburg, Germany). Antibodies originating from pooled serum of five patients with monospecific anti-PR3 antibody-positive-established WG were purified by adsorption on a PR3 affinity column as described (20). The absorbed IgG fraction (ANCA), displaying a high anti-PR3 titer, was diluted to result in final IgG concentrations of 250 ng/ml in all experiments. When preparing the F(ab)₂ fragment of ANCA (ANCA-F[ab]₂), its purity was checked to range above 95% by SDS-PAGE, and a final concentration of 250 ng/ml was used throughout. Control human IgG₁, again used at 250 ng/ml, originated from pooled serum of 100 healthy donors. Murine mAbs to human thrombomodulin were a gift from Dr. B. Pötzsch (Max-Planck-Institute, Bad Nauheim, Germany). All antibody preparations were checked for absence of endotoxin by a commercially available E-toxate assay (Sigma Chemical Co., München, Germany).

Detection of PR3 on Endothelial Cell Membrane. PR3 expression on endothelial cell surface was quantified by Cyto-ELISA with unfixed human umbilical vascular endothelial cells (HUVECs) as described (20).

Detection of PR-3 Messenger RNA in HUVECs by PCR Technique. The RNA preparation of cells was performed with the

Fast Track™ RNA-Isolation Kit (Boehringer Mannheim GmbH, Mannheim, Germany). The PR3 cDNA was synthesized by the reverse transcriptase PCR in a hybrid thermocycler (Uno-Thermoblock; Biometra, Goettingen, Germany). The sequence of the 3' primer was GCGGCGAGGGACGAAACTGCA and of the 5' primer was ATCGTGGGCGGGCAGAGGCG. The amplified cDNA had a length of 501 bp. The amplification of the PR3 cDNA was performed according to the following program: denaturation (94°C for 5 min), 30 cycles of amplification (94°C for 1 min, 58°C for 1.5 min, 72°C for 2 min), and termination at 72°C for 5 min. DNA fragments were separated in a 1.5% agarose gel.

Measurement of Endothelial Barrier Function. Albumin flux through endothelial monolayer was taken as marker of endothelial barrier function and quantified in a two-compartment system using FITC-labeled albumin as previously described (25). In separate control experiments, antibody binding to HUVEC was provoked by admixture of various concentrations of antithrombomodulin antibodies.

Analytical Procedures. For assessment of platelet-activating factor (PAF) generation, the total cellular and extracellular PAF content was lipid extracted, subjected to HPLC separation, and quantified by induction of [³H]serotonin release from prelabeled rabbit platelets as described (26). To convert [³H]serotonin release into absolute PAF concentration, calibration with exogenous PAF was performed. The phosphatidylinositol (PtdIns) turnover was investigated by measuring the accumulation of inositol phosphates according to Berridge (27). For prelabeling of cellular phospholipid pools, HUVECs were seeded on culture plates with an area of 4 cm²/well (~300 cells/mm²) in medium 199 containing 10% fetal calf serum plus 40 mM Hepes buffer, pH 7.4. Myo-[³H]inositol (5 μCi/ml) was added, and cells were incubated at 37°C for 24 h in an atmosphere of 95% O₂ and 5% CO₂. Before experimental use, cells were washed twice and kept in Hanks' balanced salt solution containing 20 mM Hepes and 10 mM LiCl. At different times after stimulus application, samples were quenched with trichloroacetic acid (final concentration 7.5%), kept on ice for 15 min, and extracted four times with diethylether. The aqueous phase was neutralized with sodium tetraborate to pH 8.0, and processed to separate inositol phosphates on Dowex anion exchange columns as described by Berridge (27). The column was eluted sequentially with water (for free [³H]inositol), 5 mM Na-tetraborate/60 mM Na-formate (for glycerophospho-[³H]inositol), 0.1 M formic acid/0.2 M ammonium formate (for [³H]IP₁), 0.1 M formic acid/0.5 M ammonium formate (for [³H]IP₂), and 0.1 M formic acid/1.0 M ammonium formate (for [³H]IP₃), and samples were processed for liquid scintillation counting.

Buffer concentrations of 6-keto-prostaglandin F_{1α}, the stable metabolite of prostacyclin, were assayed by solid phase extraction and post-HPLC ELISA as described by our laboratory (28).

Experimental Procedures. Confluent endothelial cell monolayers of the first passage were taken for these experiments. In separate control studies, fourth-passaged HUVECs were used. After removal of cell culture medium by two washing steps, priming of HUVECs with 4 ng recombinant TNF-α/ml (Boehringer Mannheim GmbH) incubated for 2 h, was performed. Cells were then washed to remove TNF, and stimulation was undertaken with MoAb-PR3 (25 ng, 250 ng, and 2.5 μg/ml), 250 ng/ml ANCA, 250 ng/ml ANCA-F(ab)₂ or 250 ng/ml control immunoglobulin. Botulinum C2 toxin, which is composed of a membrane translocation component (C_{2II}) and a component (C_{2I}) affecting ADP-ribosylation of nonmuscle G-actin, thereby acting as a barbed end-capping protein and effecting selective loss of the nonmuscle F-actin content (29), was provided by K. Aktories (Freiburg, Ger-

many). It was coapplied with antibodies at a concentration of 200 ng/ml C₂₁ and 400 ng/ml C₂₁₁. All experiments were performed in serum-free Hanks' balanced salt solution; studies with PAF measurement were undertaken in the presence of 0.25 mg/ml bovine serum albumin. Reactions were stopped by centrifugation at 4°C for 5 min at 1,200 *g*.

Statistics. For statistical comparison, one-way analysis of variance was performed. A level of $P < 0.05$ was considered to be significant.

Results

TNF preincubation of HUVECs caused a marked, time-dependent increase in the surface expression of PR3, as assessed by the binding of both monoclonal (MoAb-PR3) and affinity-purified human antibodies (ANCA) to this target (Fig. 1). An optimum TNF priming period of 2 h was identified for both antibodies. In contrast, the binding of control IgG₁ was not affected by preincubation of the endothelial cells with this cytokine.

Using PCR technique, PR3 message was detected in fourth-passaged HUVECs subsequent to TNF challenge (Fig. 2), whereas PR3 messenger RNA was not detectable in untreated HUVECs.

Corresponding with these kinetics, the sequence of TNF priming and anti-PR3 challenge caused a pronounced up-regulation of endothelial inositol phosphate formation (Figs. 3 and 4). A 2-h period was again identified as optimum TNF priming time with respect to both MoAb-PR3 and ANCA stimulation. As compared to baseline levels, the sum of sequentially formed IP₁, IP₂, and IP₃, collectively depicted as IP_x, increased up to fourfold. The antibody-

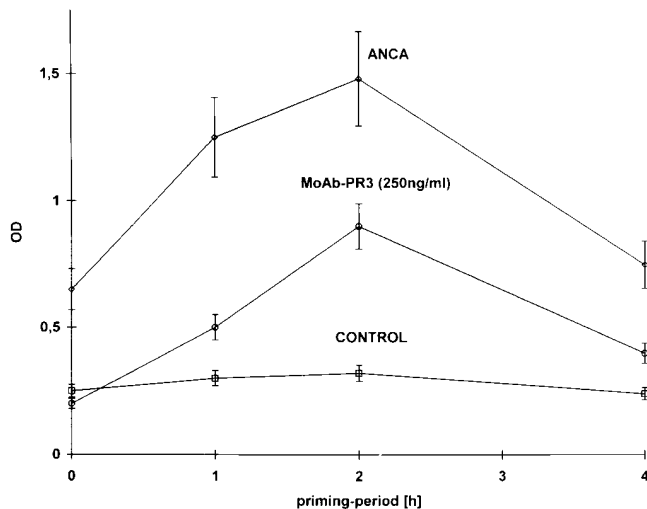


Figure 1. Influence of TNF priming on HUVEC PR3 presentation. HUVECs were incubated with TNF (4 ng/ml) for various time periods before addition of MoAb-PR3, ANCA, or control murine IgG₁. Immunoglobulin binding to the cells was assessed by Cyto-ELISA, and optical density is displayed. Mean \pm SEM of five independent experiments each is given. Experiments with ANCA and MoAb-PR3 significantly differed from the IgG₁ control.

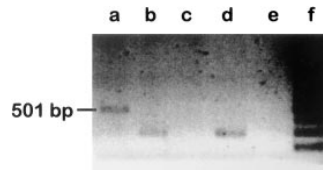


Figure 2. HUVEC PR3 messenger RNA upregulation in response to TNF priming. Priming was performed with TNF- α (4 ng/ml), for 20 min. Primed HUVECs (lane a, PR-3, 501 bp; lane b, GAPDH) express PR-3 in contrast to nonprimed cells (lane c, PR-3; lane d, GAPDH). Lane e, negative control; lane f, marker (100 bp marker from Gibco, Eggenstein, Germany).

evoked phosphoinositide hydrolysis was evident within 5 min of MoAb-PR3 and ANCA admixture, and the maximum response occurred after 20 min, with subsequent decline of IP_x levels. The F(ab)₂ fragment of ANCA displayed virtual identical efficacy as ANCA itself. In contrast, control murine and human IgG was completely ineffective. In separate experiments, fourth-passaged HUVECs were used and corresponding PI hydrolysis in response to anti-PR3 was obtained (Fig. 5).

Coexposure of HUVECs to MoAb-PR3 and botulinum C2 toxin did not suppress the phosphoinositide response. In experiments performed in one batch of endothelial cells, 25 ng/ml MoAb-PR3 elicited 725.3 ± 52.4 cpm (10 min) and 830 ± 51.0 cpm (20 min) IP_x in the absence of C2 toxin, as compared to 839 ± 34.5 cpm (10 min) and $1,014 \pm 62.1$ cpm (20 min) in the presence of the toxin.

In parallel with the phosphoinositide hydrolysis, endo-

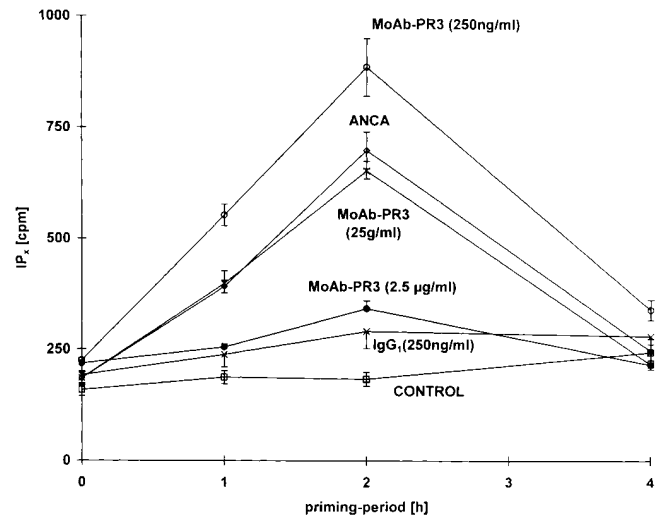


Figure 3. Influence of TNF-priming period on HUVEC activation by anti-PR3 challenge. HUVECs were incubated with TNF (4 ng/ml) for various time periods before addition of MoAb-PR3, ANCA, murine control IgG₁ (IgG₁), or sham incubation (CONTROL). IP formation was assessed 20 min after onset of antibody incubation. Mean \pm SEM of five independent experiments each is given. Experiments with ANCA and MoAb-PR3 significantly differed from control. In additional control experiments with nonspecific human IgG, data (not displayed) did not differ from control.

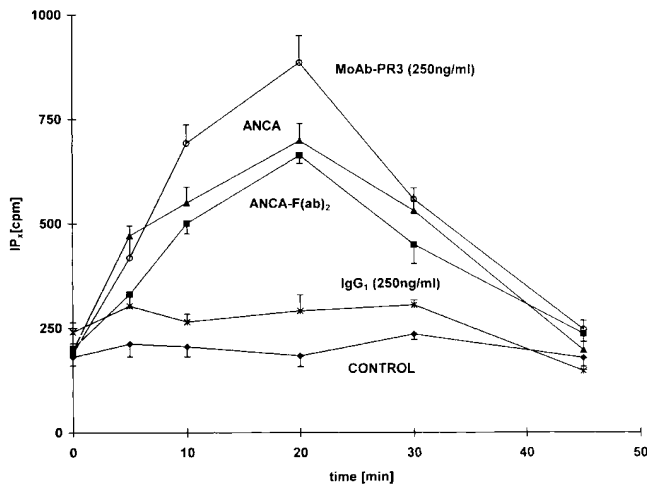


Figure 4. Time course of IP accumulation in response to anti-PR3 challenge. After a priming period of 2 h with TNF (4 ng/ml), HUVECs were challenged with MoAb-PR3, ANCA, ANCA-F(ab)₂, or murine control IgG₁ (IgG₁) for various time periods, or sham incubation was performed (CONTROL). Data are given as mean ± SEM of five independent experiments. Experiments with ANCA, ANCA-F(ab)₂, and MoAb-PR3 significantly differed from control. In additional control experiments with nonspecific human IgG, data (not displayed) did not differ from control.

thelial PAF generation was provoked by the sequence of TNF priming and anti-PR3 challenge (Fig. 6). The maximum metabolic response again occurred 20 min after antibody admixture; control IgG was completely ineffective.

Similarly, impressive stimulation of prostacyclin synthesis was noted in TNF-primed endothelial cells undergoing challenge with MoAb-PR3 (Fig. 7) and ANCA (data not given in detail), but not with control IgG. As anticipated, the prostanoid release reaction was completely abrogated in the presence of acetylsalicylic acid.

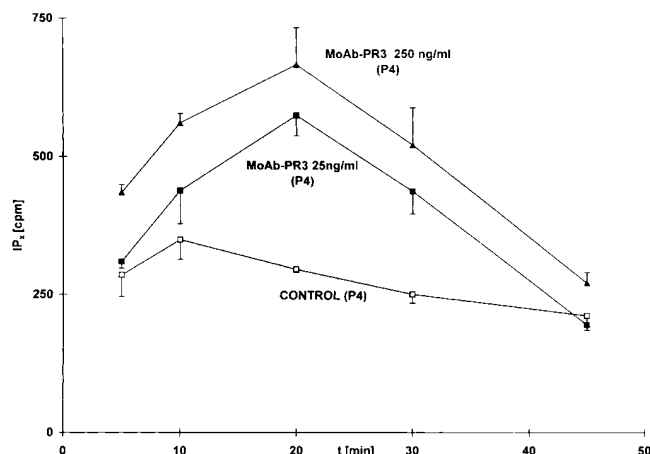


Figure 5. Effect of anti-PR3 on IP formation in fourth-passaged HUVECs. After a priming period of 2 h with TNF (4 ng/ml), HUVECs of subculture 4 (P4) were challenged with different concentrations of MoAb-PR3 for various time periods or sham incubated (CONTROL). Data are given as mean ± SEM of five independent experiments. Experiments with the different concentrations of MoAb-PR3 significantly differed from control.

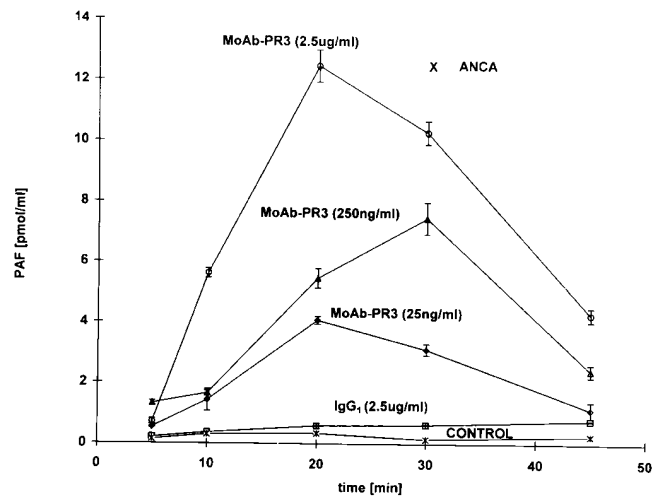


Figure 6. Time and dose dependency of PAF synthesis in response to anti-PR3 challenge. After 2 h of TNF priming, HUVECs were challenged with different concentrations of MoAb-PR3, ANCA (only one time point), or control murine IgG₁. PAF synthesis is given in pmol PAF/ml assay volume. Mean ± SEM of five independent experiments each is depicted. Experiments with the different concentrations of MoAb-PR3 significantly differed from control IgG₁.

The sequence of cytokine priming and anti-PR3 challenge resulted in a marked loss of endothelial barrier properties (Fig. 8). Stimulation of the monolayer with MoAb-PR3 in the absence of TNF preincubation did not affect the baseline albumin passage. TNF priming per se, followed by stimulation with control IgG, resulted in some marginal increase in protein flux. This was significantly increased when TNF-primed HUVECs were incubated with anti-PR3 mAbs. The time course of this response was, however, slower compared to the metabolic events; the

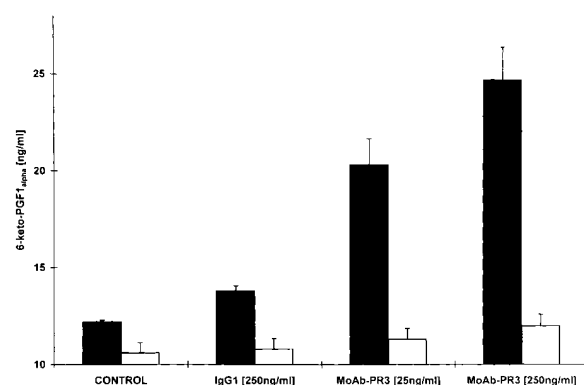


Figure 7. Prostaglandin I₂ formation in response to MoAb-PR3. After 2 h of TNF priming, HUVECs were challenged with different concentrations of MoAb-PR3, control murine IgG₁, or were incubated in the absence of immunoglobulins (CONTROL). Prostanoid release into the cell supernatant was quantified 20 min after antibody admixture. Gray columns represent parallel samples preincubated with acetylsalicylic acid (10 μM) for 5 min. Mean ± SEM of five independent experiments each is given. Experiments with MoAb-PR3 in the absence of acetylsalicylic acid significantly differed from control and nonspecific murine IgG₁.

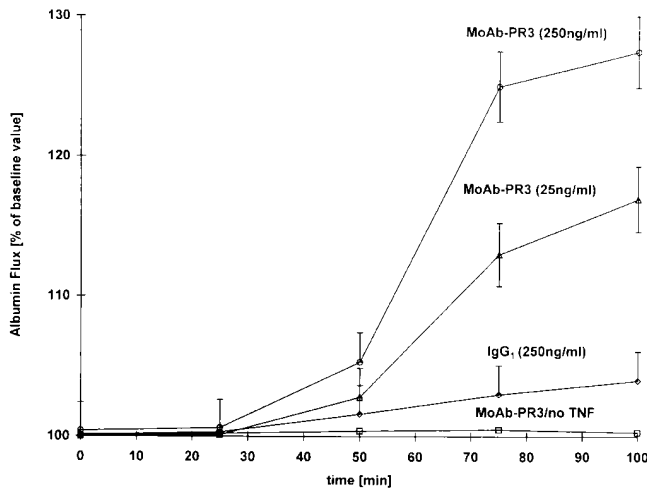


Figure 8. Time and dose dependency of anti-PR3 induced loss of endothelial barrier function. After 2 h of TNF priming, HUVECs were challenged with different concentrations of MoAb-PR3 or control murine IgG₁. In separate control experiments, MoAb-PR3 (250 ng/ml) was admixed to HUVECs undergoing sham priming in the absence of TNF. Albumin flux through the endothelial monolayer is displayed in percent of baseline value; 100% corresponds to permeability of $(9.8 \pm 0.75) \times 10^{-6}$ cm/s of barrier formed by TNF-primed HUVEC monolayer and supporting filter membrane and to $(8.3 \pm 0.58) \times 10^{-6}$ cm/s to barrier formed by untreated HUVEC monolayer and supporting filter membrane. Mean \pm SEM of five independent experiments each is given. The experiments with 250 ng/ml and 25 ng/ml MoAb-PR3 significantly differed from both control groups.

onset of leakage was evident after 50 min, and progressive deterioration of barrier properties occurred up to the end of the observation period (100 min). In separate control experiments performed to probe the effect of non-PR3-related surface antigen binding on permeability characteristics of the endothelial monolayer, untreated HUVECs were exposed to murine antithrombomodulin mAbs in various concentrations (25 ng–25 μ g/ml). This antibody is known to bind to thrombomodulin, present in abundance on the cell surface of HUVECs (30, 31). None of these experimental conditions provoked any significant change in albumin flux. In detail, 100 min after antibody admixture, albumin flux was $103 \pm 8.8\%$, $105 \pm 6.4\%$, $97 \pm 10.1\%$, and $101 \pm 9.6\%$ of baseline in response to 25 ng/ml, 250 ng/ml, 2.5 μ g/ml, and 25 μ g/ml antithrombomodulin antibody, respectively. The lack of HUVEC responsiveness to antithrombomodulin antibodies was equally observed in cells pretreated with 4 ng TNF/ml according to the standard protocol.

All MoAb-PR3-induced metabolic changes as well as the leakage response displayed a maximum upon use of 250 ng/ml mAb, with lower efficacy of both 25 ng and 2.5 μ g/ml. In contrast, PAF synthesis was found to be maximal at 2.5 μ g/ml MoAb-PR3. This difference is most probably explained by the fact that albumin was admixed to the medium in case of PAF measurement, thus reflecting antibody binding by the serum protein.

Discussion

It has previously been shown that priming of human neutrophils with cytokines such as TNF- α induces a translocation of PR3 from the azurophilic granules to the cellular surface (10–13). In the case of endothelial cells, however, the origin of PR3 is much less obvious. Circulating free PR3 has been shown to bind to the surface of these cells (32); however, all solutions currently used were tested to be devoid of PR3, including the cell culture medium. PR3 was shown to be present in the cytoplasm of untreated cultured endothelial cells by confocal laser scanning microscopy (20), and in correspondence with this preceding study the current Cyto-ELISA data, using two different specific anti-PR3 antibodies, leave no doubt that the TNF exposure results in endothelial surface expression of this antigen. In addition, TNF-elicited appearance of PR3 message in the endothelial cells was demonstrated in this study.

When compared to studies with antiendothelial antibodies purified from scleroderma patients (33, 34), which used antibody doses of ~ 100 μ g/ml to effect endothelial cell activation, strikingly low concentrations of ANCA (250 ng/ml) and MoAb-PR3 (25 ng–2.5 μ g/ml) currently sufficed to provoke marked signaling and metabolic events in the human endothelial cells. The phosphoinositide hydrolysis affected by both antibodies peaked in parallel with the TNF-evoked presentation of PR3, and its magnitude approached that known to be provoked by thrombin, the most potent activator of the phosphatidylinositol response in endothelial cells hitherto described (35). Via IP₃-affected calcium liberation, this signaling sequence is linked to activation of phospholipolytic pathways with subsequent generation of prostacyclin and PAF. It is in line with this reasoning that the time course of both prostanoid and PAF synthesis paralleled the kinetics of phosphoinositide hydrolysis in the current study.

The link between antibody binding and triggering of phosphoinositide hydrolysis is, however, much less obvious. Receptor/antigen cross-linking and the involvement of Fc receptors may be excluded, as the F(ab)₂ fragment of ANCA turned out to be equally potent as ANCA itself. This is also true for complement receptors, as the experiments were performed in the absence of complement sources. Moreover, phenomena of internalization of the antigen-antibody complex are unlikely to be enrolled, as disruption of the endothelial F-actin content with botulinum C2-toxin (36) did not suppress the phosphoinositide hydrolysis. Thus, monomeric IgG ligand binding must be assumed to suffice for signal transduction. Interestingly, activation of phosphoinositide hydrolysis was not observed after antithrombomodulin challenge used as control antibody targeting an abundant endothelial surface antigen. Further studies are required to elucidate the mechanism by which the anti-PR3 binding is linked to phospholipase C activation and thus phosphatidylinositol hydrolysis.

In addition to the metabolic responses, incubation of human endothelial cells with anti-PR3 antibodies evoked a marked increase in transendothelial protein leakage. Such

loss of barrier properties has previously not been described for antiendothelial antibodies encountered in human disease. The leakage response presented with an identical dosage optimum of MoAb-PR3 as the phosphoinositide hydrolysis, thus suggesting a link between these two events. It did, however, demand a much longer latent period (>30 min) as compared to the IP₃ accumulation (maximum after 20 min), suggesting that it is not simply linked to IP₃-related calcium increase and associated rapid contractile events (37). This provocative part of the endothelial response to anti-PR3 challenge thus also demands further elucidation.

In conclusion, priming of human endothelial cells with TNF and exposure to specific anti-PR3 antibodies is capable of triggering a central signaling cascade in these cells, in companion with lipid mediator generation and loss of endothelial barrier properties. These findings refute the ex-

clusiveness of PMNs and monocytes as targets of anti-PR3 in instrumenting vascular injury in WG. The preconditions for endothelial cell activation and leakage response, being the presence of both autoantibodies and circulating cytokines, may well occur *in vivo* under circumstances of infection or due to the autoantigenic process itself once it is triggered. In accordance, TNF, interleukin 6, and interleukin 8 levels are known to be elevated in WG patients in active disease, but are not detectable in remission (38, 39). The current findings, identifying endothelial cells as active participants rather than innocent bystanders in the scenario of leukocyte-endothelium interaction, further support and substantially extend the concept that c-ANCA represent not only the best seromarker for WG so far available, but are centrally involved in the pathogenic sequelae of this enigmatic disease.

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