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Interaction of Antibodies to Proteinase 3 (Classic Anti-Neutrophil Cytoplasmic Antibody) with Human Renal Tubular Epithelial Cells: Impact on Signaling Events and Inflammatory Mediator Generation

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Among the anti-neutrophil cytoplasmatic Abs (ANCA), those targeting proteinase 3 (PR3) have a high sensitivity and specificity for Wegener’s granulomatosis (WG). A pathogenetic role for these autoantibodies has been proposed due to their capacity of activating neutrophils in vitro. Recently, PR3 was also detected in human renal tubular epithelial cells (TEC). In the present study, the effect of murine monoclonal anti-PR3 Abs (anti-PR3) and purified c-ANCA targeting PR3 from WG serum on isolated human renal tubular cell signaling and inflammatory mediator release was characterized. Priming of TEC with TNF-α resulted in surface expression of PR3, as quantified in immunofluorescence studies and by flow cytometry. Moreover, PR3 was immunoprecipitated on surface-labeled TEC. Primed TEC responded to anti-PR3 with a dose- and time-dependent activation of phosphoinositide hydrolysis, resulting in a remarkable accumulation of inositol phosphates. Control IgG was entirely ineffective, whereas PR3-ANCA reproduced the phosphoinositide response. The signaling response was accompanied by a pronounced release of superoxidanion into the cell supernatant. Moreover, large amounts of PGE2 and, to a lesser extent, of thromboxane B2, the stable metabolite of TxA2, were secreted from anti-PR3-stimulated TEC. In parallel, a rise in intracellular cAMP levels was observed, which was blocked by the cyclooxygenase inhibitor indomethacin. We conclude that anti-PR3 Abs directly target renal TECs, thereby provoking pronounced activation of the phosphoinositide-related signal transduction pathway. Associated metabolic events such as the release of reactive oxygen species and lipid mediators may directly contribute to the development of renal lesions and loss of kidney function in WG. The Journal of Immunology, 2002, 168: 3057–3064.

Wegener’s granulomatosis (WG) is characterized by systemic vasculitis affecting predominantly the respiratory tract and the kidneys. The development of renal lesions represents a limiting factor for the course and prognosis of this disease (1, 2). Morphologically, as assessed in renal biopsies, necrotizing, crescentic pauci-immune glomerulonephritis, and marked leukocyte influx into glomeruli and interstitium represent the predominant findings (3–5). Cells accumulating in the extracapillary space of the glomeruli and around tubuli include neutrophils, monocytes, lymphocytes, and epithelial cells. Recent evidence suggests that tubular epithelial cells (TEC) play an active role in the development of tubulo-interstitial injury in glomerulonephritis, as TEC were noted to be capable of releasing proinflammatory cytokines and of expressing adhesion molecules (6–10).

The pathogenetic events underlying the development of the complex renal lesions in WG are, however, still poorly defined. The diagnosis of WG has largely profited from the detection of anti-neutrophil cytoplasmatic Abs (ANCA) (11). Proteinase 3 (PR3), a leukocyte serine protease localized within the granules of neutrophils and monocytes, was identified as the main target Ag for ANCA, producing a cytoplasmatic staining on ethanol-fixed neutrophils (c-ANCA) (12–14). c-ANCA targeting PR3 do possess a high sensitivity and a nearly 95% specificity for WG (15–17). Besides representing a seromarker, with the autoantibody titer correlating with disease activity in vivo (11, 17), there is now ample evidence that ANCA are involved in the pathogenesis of WG. ANCA are capable of stimulating human neutrophils, provoking a respiratory burst, degranulation, and release of lipid mediators and cytokines in vitro (18–23). Moreover, activated neutrophils have been detected within kidney biopsies of WG patients, with the amount of infiltrating leukocytes corresponding to disease activity (24). Thus, ANCA-induced neutrophil activation appears to be centrally involved in the development of renal lesions characterizing WG.

However, recent findings suggest that leukocytes are not the exclusive target cells for ANCA binding. PR3 was detected in renal biopsies of WG patients, with tubular casts being clearly positive for this proteinase (24, 25). Moreover, the presence of PR3-specific mRNA was detected in cultured cytokine-treated human endothelial and renal TECs (26, 27), as well as in kidney biopsies, with distal tubules as well as glomerular epithelia being strongly positive for PR3 message (28). While binding of c-ANCA to endothelial cells is followed by expression of adhesion molecules...
(29) and activation of signaling responses such as phosphoinosi-
tide hydrolysis with progressive loss of endothelial barrier prop-
ties (30), the effects of c-ANCA interaction with renal TECs are
largely unknown. Such interaction may, however, be of major
interest, as renal TEC may well be involved in inflammatory
events, by expression of adhesion molecules, promotion of leukocyte
recruitment, and synthesis of inflammatory mediators (6–10). Tu-
bular-derived lipid mediators such as PGs and thromboxane (Tx)
can cause severe vasoregulatory disturbances, thereby contributing
to the impairment of renal function (31–33). Moreover, TEC can
produce a variety of reactive oxygen metabolites, such as superoxide
anion (O2−), which may aggravate renal tissue damage (34, 35).

Hypothesizing that the interaction of c-ANCA with tubular ep-
thelium contributes to the development of renal lesions associated
with WG, we now studied the effects of murine monoclonal anti-
PR3 Abs (anti-PR3) and anti-PTR3 Abs purified from WG sera
(PR3-ANCA) on signaling events and inflammatory mediator gen-
eration in human renal TEC in vitro. In essence, marked stimu-
lization of phosphoinositide hydrolysis-related signaling events was
noted, associated with superoxide release and prostanoid genera-
tion. TECs may thus well play an active role in the pathogenesis of
renal lesions in WG.

Materials and Methods
Isolation and culture of TEC

TEC were isolated from human kidney portions obtained from nephrecto-
 mies, as originally described by van der Biest et al. (36) and adapted by
Schwarting et al. (27). The macroscopically normal tissue was dissected
and immediately transferred to ice-cold medium M199 (Seromed, Berlin,
Germany) containing 10% FCS (Life Technologies, Eggenstein, Ger-
many). TEC were isolated by passing the tissue through a 100-μm
mush. The tissue was dissected and discarded, and the outer medulla was cut into pieces of 2–
3 mm3. After incubation with HBSS
(Life Technologies) containing 0.1% collagenase type II (Seromed) for 30
min at 37°C with gentle shaking, the tissue was passed through a 120-μm
mush. The resulting suspension was centrifuged with a Metrazimide gra-
dient (Nycoderm, Oslo, Sweden). All material from the top of the gradient
was collected, washed twice, and finally resuspended in M199 containing
20 FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml t-
glutamine, 17 U/ml heparin, and 20 μg/ml endothelial cell growth factor
(Boehringer Mannheim, Mannheim, Germany). Cells were placed into 25-
cm2 gelatin-coated culture flasks (Costar, Badenheim, Germany) and
and grown at 37°C in an atmosphere of 95% O2 and 5% CO2. Confluent mono-
layers (average growth of 7 days) were subcultured by trypsin treatment
(trypsin 0.25%, EDTA 0.04%; Life Technologies) with a split ratio of 1:3.
For experimental use, cells were plated on gelatin-coated 12-well multi-
wells (4 cm2/well, ~300 cells/mm2; Costar).

Identification of TEC

Cells of subcultures 1 and 2 were used for immunohistochemical staining
and characterized as previously described (27). In brief, the epithelial or-
igin of these cells was confirmed by using Abs to cytokeratin 8 and cyto-
keratin 19 (Novocastra, Newcastle, Germany), whereas Abs to GST-α (Biotrin, Dublin, Ireland), and γ-glutamyl
transferase (mAb 102 D2, K2, B11, kindly provided by Dr. N. Sabo-
lovic, Nancy, France) were used to identify proximal TEC. Contamination
with endothelial cells (factor VIII-related Ab), monocytes (CD45, CD14),
and fibroblasts (5SBS) was excluded.

The relative percentage of proximal and distal TEC was determined
by MICS using mAb 102 D2, K2, and B11 and Abs to HMFG1, HMFG2,
GST-α, and GST-α according to standard methods. MICS analysis was
performed in a FACScan (BD Biosciences, Mountain View, CA) using
forward and orthogonal light scatter to select viable cells. Data for 3000
cells were collected. Ninety-eight percent of isolated cells were identified
as epithelial cells, with >85% of TEC as distal TEC according to MICS
analysis (data not shown).

Determination of PR3 surface expression by indirect
immunofluorescence

For the determination of PR3 surface expression, immunofluorescence
studies with unfixed TEC of subcultures 1 and 2 were performed. Cells
were seeded on gelatin-coated eight-well chamber slides (Lab-Tek; Miles
Scientific, Naperville, IL). After the removal of cell culture medium, con-
fluent cells were incubated with TNF-α (4 ng/ml; Boehringer Mannheim)
for various time periods. Subsequently, TNF was removed and 100 μg/ml
pooled human IgG (Octagam; Octapharma, Langenfeld, Germany) was
added to block nonspecific binding of anti-PR3. Then, TEC were incubated
with murine monoclonal anti-PR3 Abs (10 μg/ml) or isotype-matched con-
trol mouse IgG (IgG) (10 μg/ml, MOPC-21; Sigma, Deisenhofen,
Germany) for 30 min. After two washings with PBS, the secondary Ab, a FITC-
conjugated rabbit anti-mouse IgG (diluted 1/200) (F-0261; DAKO) was
added and again incubated for 30 min. Stained cells were analyzed with a Zeiss
axiophot microscope (Zeiss, Oberkoche, Germany).

Determination of PR3 surface expression by flow cytometry

To further confirm the immunofluorescence data, PR3 surface expression
was assessed by flow cytometry. Therefore, TNF-primed TEC of subcul-
ture 1 or 2 were distributed to flexible round-bottom microtiter plates (2 x
105 cells/well). Before the addition of the different monoclonal anti-PR3
Abs (WGM, A43, A45, and 12.8), 20 μl of pooled human IgG (100 μg/ml;
Octapharma) were added to block Fe IgGRs. Next, 20 μl of murine mono-
clonal anti-PR3 Abs (10 μg/ml) or mouse control IgG (10 μg/ml, MOPC-
21) were added, and incubation was performed for 30 min at 4°C. After
three washes, the secondary Ab, a PE-conjugated goat anti-mouse IgG (50
μg/ml; DAKO) was added and again incubated for 30 min at 4°C. After
three more washes, cells were resuspended in PBS (0.1% sodium
acetate).

Flow cytometry was performed in a FACScan (BD Biosciences) using forward
and orthogonal light scatter to select viable cells. CellQuest research software
(BD Biosciences) was used for analysis of data.

Determination of PR3 expression by immunoprecipitation

Immunoprecipitation was performed as recently described by Bux et al.
(37). In brief, TNF-primed, unfixed TEC were biotinylated (5 mmol/L
NHS-LC-Biotin; Pierce, Rockford, IL) for 30 min on ice. After two
washes, monoclonal anti-PR3 Abs (A43 and WGM), or mouse control IgG
(MOPC-21), each at 10 μg/ml, were added to the cell suspensions (1 x
107 cells/sample) and incubated for 30 min at 37°C. Cells were washed and
lysed by adding lysis buffer (1% Triton X-100, 5 mmol/L EDTA, 2
mmol/L PMSF, 0.5 μg/ml leupeptin, 500 μmol aspirin in 20 mmol/L
Tris-buffered saline (pH 7.4)) for 30 min at room temperature. After son-
ication and centrifugation, supernatants were incubated with rabbit anti-
mouse IgG Abs (DAKO) coupled to protein A-Sepharose CL-4B (Phar-
macia, Uppsala, Sweden). The protein A-Sepharose beads were washed and
resuspended in SDS-PAGE sample buffer, boiled, and then subjected to
10% SDS-PAGE. After electrophoresis, proteins were transferred onto
nitrocellulose (Hybond C; Amersham, Braunschweig, Germany). For vis-
ualization, the nitrocellulose was first blocked with 1% BSA (Sigma) in
PBS and then incubated with streptavidin conjugated to peroxidase (Zymed
Laboratories, San Francisco, CA). Unbound streptavidin was washed out
and the nitrocellulose was incubated with a chemiluminescent substrate
(ECL; Western Blotting Detection System; Amersham) and then exposed to
film. Molecular mass of the detected protein was calculated using
two batches of molecular mass standard (Kaleidoscope prestained stan-
dards from Bio-Rad (München, Germany) and RPN 800 from Amersham).

Experimental protocol

Experiments were performed with confluent monolayers of subcultures 1 and
2. After removal of cell culture medium, cells were washed twice and
kept in HBSS containing 20 mM HEPEs. Priming was performed with 4
μg/ml TNF for 2 h. Before adding of murine monoclonal anti-PR3 Abs,
PR3-ANCA from WG sera, normal human IgG (Octagam; Octapharma),
isotype-matched mouse control IgG (MOPC-21; Sigma), or buffer (control)
cells were washed twice to remove TNF. When indicated, arachidonic acid
(AA; 5 μM; Sigma) was added simultaneously with the Abs. Whenever
necessary, indomethacin (10 μM; ICN Biomedicals, Aurora, OH) was
added 10 min before Ab challenge. Reactions were stopped by addition of
7.5% trichloroacetic acid (phosphatidylinositol turnover) or 70% ethanol
cAMP levels), or at 4°C (O2 and prostanoid generation).

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**Phosphoinositide hydrolysis**

The phosphatidylinositol turnover was investigated by measuring the accumulation of inositol phosphates (IPs) according to Berridge (38). For prelabeling of cellular phospholipid pools, Myo-[\(^{3}H\)]inositol (5 \(\mu Ci\)/well; Amersham, Dreieich, Germany) was added to the confluent monolayers, and cells were incubated at 37°C for 24 h in an atmosphere of 95% O_2 and 5% CO_2. Before experimental use, cells were washed twice and kept in HBSS containing 20 mM HEPES and 10 mM LiCl. At different times after stimulus application, samples were quenched with trichloroacetic acid (v/v; 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 and processed to separate IPs on Dowex anion exchange columns as described by Berridge (38). The column was eluted sequentially with water (for free [\(^{3}H\)]inositol), 5 mM Na-tetraborate/60 mM Na-formate (for glycerophospho-[\(^{3}H\)]inositol), 0.1 M formic acid/0.2 M ammonium formate (for [\(^{3}H\)]IP), 0.1 M formic acid/0.5 M ammonium formate (for [\(^{3}H\)]IP), and 0.1 M formic acid/l M ammonium formate (for [\(^{3}H\)]IP)., and samples were processed for liquid scintillation counting.

**Superoxide anion (O\(_{2}^{\cdot}\)) release**

TEC O\(_{2}^{\cdot}\) generation was measured as superoxide dismutase-inhibitable reduction of cytochrome c. In brief, duplicate reaction mixtures containing TEC, and 75 \(\mu M\) ferricytochrome c (Sigma) were incubated at 37°C in the presence or absence of 10 \(\mu g/ml\) superoxide dismutase (Sigma). Stimuli were coapplied with cytochrome c. Incubations were terminated by centrifugation at 4°C at 1200 \(\times\) g. O\(_{2}^{\cdot}\) release was quantitated as nanomoles of cytochrome c reduction, using an extinction coefficient of 21 M \(^{-1}\) cm \(^{-1}\) at 550 nm in a Uvicom spectrophotometer (Uvicom, Zurich, Switzerland).

**Lipid mediator generation and intracellular cAMP levels**

For quantitation of lipid mediator release, reactions were stopped at 4°C and cell supernatants were collected and stored at -20°C until further processing. Cyclic nucleotides were extracted from cellular pools in 70% ice-cold ethanol for 1 h and extracts were evaporated to dryness under a continuous nitrogen stream and stored at -70°C.

Release of PGE\(_{2}\) and TxB\(_{2}\), the stable metabolite of TxA\(_{2}\), into the cell supernatant was quantitated by commercial ELISA kits (Cayman Chemicals, Ann Arbor, MI), and cAMP was analyzed using a commercial RIA system (Amersham).

**Preparation of anti-PR3 Abs**

Murine monoclonal anti-PR3 Abs as well as affinity-purified PR3-ANCA from WG sera were kindly provided by Dr. E. Csernok (Rheumaklinik Bad, Bramsted, Germany) and prepared as previously described (12). In brief, murine monoclonal anti-PR3 Abs were prepared by hybridoma technology, and the clone WGM\(_{i}\) (IgG1) was chosen for additional experiments. PR3-ANCA originating from pooled serum of patients with monospecific anti-PR3 Ab-positive established WG were purified by adsorption to a PR3 affinity column. PR3 specificity of the monoclonal and serum-derived Abs was assessed in a commercial Ag-specific ELISA (Or- gentec, Mainz, Germany). Endotoxin contamination of the murine and human anti-PR3 Abs was below 15 pg/ml, as assessed by the kinetic-OLC Luminis amebocyte cell lysis test (Chromogenix, Mölndal, Sweden). The monoclonal anti-PR3 Abs 4A5 and 4A3 were purchased from Wieslab (Lund, Sweden), whereas the anti-PR3 Ab 12.8 was from Research Diagnostics (Flanders, NY).

**Statistics**

For statistical comparison, one-way ANOVA was performed, followed by Tukey’s honestly significant difference test when appropriate. A level of \(p < 0.05\) was considered significant.

**Results**

**PR3 surface expression by TEC**

Because Schwarting et al. (28) could previously demonstrate that human TEC are capable of de novo PR3 synthesis, we were now focusing on the surface expression of the c-ANCA autoantigen on the tubular cells. Renal tubular PR3 surface expression was determined in immunofluorescence studies using unfixed TEC. On TNF-pretreated TEC, but not on unprimed cells, addition of murine monoclonal anti-PR3 Abs (10 \(\mu g/ml\)) produced a strong diffuse staining on unfixed TEC (Fig. 1B) as compared with staining by isotype-matched control Abs (10 \(\mu g/ml\)) (Fig. 1A). To further confirm the immunofluorescence data, flow cytometry was performed, using WGM\(_{i}\), and three additional monoclonal anti-PR3 Abs, with one Ab (4A5) recognizing the same epitope of PR3 as WGM\(_{i}\) and two other Abs (4A3 and 12.8) directed against different epitopes of the c-ANCA target Ag (39). As depicted in Fig. 2, all monoclonal anti-PR3 Abs produced a positive staining on unfixed, TNF-treated TEC, as compared with staining by isotype-matched control IgG. Finally, to ensure that the anti-PR3 Abs indeed recognized PR3 on the surface of TEC, surface labeling of TEC, followed by immunoprecipitation, was performed. These experiments showed that two different anti-PR3 Abs, but not isotype-matched control IgG, specifically bound to the surface of the TNF-primed TEC, with both 4A5 and WGM\(_{i}\) precipitating a protein of the expected molecular mass of PR3 (29 kDa) (Fig. 3).

Because maximal PR3 expression was noted after a 2-h pre-treatment with 4 ng/ml TNF, the following experiments were performed under these priming conditions.

**Anti-PR3-induced activation of tubular phosphoinositide hydrolysis**

Whereas unprimed TEC were not activated by monoclonal anti-PR3 Abs, the sequence of TNF priming and anti-PR3 challenge provoked a pronounced time- and dose-dependent activation of tubular inositol phosphate formation (Fig. 4). After 15 min of stimulation, the sum of sequentially formed IP\(_g\), IP\(_g\), and IP\(_g\), collectively depicted as IP\(_g\), increased to 256 ± 7% of baseline levels upon challenge with 2.5 \(\mu g/ml\) anti-PR3, with lower efficacy of 250 ng/ml anti-PR3 (184 ± 8% of baseline). The extent of the signaling response thus corresponds well to IP\(_g\) formation elicited by 10\(^{-7}\) M bradykinin (289 ± 22% of baseline), one of the most
potent activators of tubular phosphoinositide hydrolysis. The autoantibody-evoked IP formation peaked after 15 min, with a rapid decline thereafter. Activation of phosphoinositide hydrolysis could be reproduced with PR3-ANCA (2.5 g/ml), whereas corresponding amounts of murine and human control IgG turned out to be completely ineffective.

FIGURE 2. PR3 surface expression on cultured TEC (flow cytometry). After a priming period of 2 h with TNF-α (4 ng/ml), unfixed TEC were incubated with the anti-PR3 Abs WGM2 (A), 4A5 (B), 4A3 (C), or 12.8 (D), or with an isotype-matched control IgG for 30 min. All Abs were used at 10 μg/ml. After three washings, a PE-labeled goat anti-mouse IgG (50 μg/ml) was added and further incubated for 30 min. After three further washings, flow cytometry was performed. Note the positive staining for PR3 produced by all mAbs (open histograms) vs control IgG (filled histograms). Representative data of three different experiments are given.

O₂⁻ generation
The anti-PR3-evoked signaling response was accompanied by marked O₂⁻ formation (Fig. 5). In TNF-primed TEC, anti-PR3 challenge provoked a time- and dose-dependent secretion of O₂⁻ with values increasing up to six times over baseline. Superoxide formation became apparent within 15 min after autoantibody admixture and peaked after 30 min of incubation. Again, 2.5 μg/ml anti-PR3 displayed a higher efficacy than 250 ng/ml anti-PR3, and incubation with isotype-matched control IgG did not elicit any substantial release of O₂⁻.

Lipid mediator release
Being a characteristic metabolic property of distal TECs, the generation of the cyclooxygenase (COX) metabolites of AA, PGE₂, and TxA₂ was investigated in anti-PR3-challenged TEC. While anti-PR3 as a sole stimulus provoked only the liberation of some minor quantities of these lipid mediators (data not shown), simultaneous addition of exogenous AA (5 μM) resulted in a prominent release of PGE₂ and, to a lesser extent, TxB₂, the stable metabolite of TxA₂, into the cell supernatant of anti-PR3-stimulated cells (Fig. 6). Application of sole AA resulted in a 2-fold increase, while 2.5 μg/ml anti-PR3 plus AA elicited an ~6-fold increase in PGE₂ and TxB₂ over controls. Lipid mediator release peaked after 30 min of incubation, with 2.5 μg/ml anti-PR3 being more efficient than 250 ng/ml anti-PR3. Again, incubation of TEC with isotype-matched control IgG plus AA did not result in any activation of tubular COX metabolite formation. As expected, anti-PR3-induced prostanoid release was completely blocked in the presence of indomethacin (10 μM) (Fig. 7).

Intracellular cAMP levels
Because PGE₂, the predominant renal tubular COX product elicited by anti-PR3 challenge, is a potent activator of adenylatecyclase, the effect of anti-PR3 on tubular cAMP levels was studied. Anti-PR3 challenge plus AA supply elicited a time- and dose-dependent elevation of intracellular cAMP levels, which succeeded the Ab-induced liberation of PGE₂ (Fig. 8). As anticipated from the data in PGE₂ synthesis, anti-PR3, as a sole stimulus, did not induce any substantial elevations of cAMP levels (data not shown), whereas in the presence of AA a remarkable elevation of cAMP in TEC challenged with anti-PR3 was observed. The levels of cAMP remained unchanged in TEC incubated with sole AA or with isotype-matched control IgG plus AA. The anti-PR3-induced rise in tubular cAMP content was completely blocked in the presence of indomethacin (Fig. 9).

Discussion
The data presented in this study further support the concept that renal TECs represent direct target cells for PR3-ANCA, with surface expression of PR3 on these epithelial cells being up-regulated under inflammatory conditions such as the presence of TNF-α. Under these conditions, PR3-ANCA purified from WG serum and murine mAbs to PR3, but not the respective control Igs, caused
marked activation of phosphoinositide hydrolysis-related signaling events in the TEC. Additionally, epithelial superoxide generation and marked liberation of prostanoids was provoked. The latter are suggested to increase the epithelial cAMP content in an autocrine fashion. Direct targeting of renal TECs by Abs to PR3 thus provokes complex metabolic events in this cell type.

While it is well accepted that PR3 is present on the plasma membrane of cytokine-primed neutrophils and monocytes (12, 40, 41), the capacity of nonhematopoietic cells to express the c-ANCA target Ag has been the topic of controversial discussions (26, 27, 42–45). In previous studies using cryostat sections from human kidneys (24, 25), PR3 was found to be present in tubular casts as well as within TECs, although it was assumed that PR3 positivity of TEC might be due to the uptake of the cationic protein by these cells. Recently, however, PR3 message and surface expression have been detected in cultured distal TEC, and the interaction of c-ANCA and tubules induced an up-regulation of tubular adhesion molecule expression (27). Moreover, PR3-mRNA and protein were recently detected in kidney biopsies by in situ hybridization techniques, with distal tubules as well as glomerular epithelia being strongly positive for PR3 message (28). In addition, glomerular

FIGURE 4. Time course of inositolphosphate accumulation in response to anti-PR3 challenge. After a priming period of 2 h with TNF-α (4 ng/ml) or sham priming, TEC were challenged with purified c-ANCA targeting PR3 from WG sera (PR3-ANCA; 2.5 μg/ml), murine monoclonal anti-PR3 Abs (anti-PR3; 2.5 μg/ml and 250 ng/ml), normal human IgG (IgGh; 2.5 μg/ml) or isotype-matched control mouse IgG (IgGm; 2.5 μg/ml) for various time periods. Extracted inositolphosphates (IP₁, IP₂, IP₃) are collectively depicted as IPₓ and expressed as the percentage of baseline levels (TNF-primed, sham-stimulated cells). Means ± SEM of at least four independent experiments each are given. *, Values differ significantly from control IgG (p < 0.05).

FIGURE 5. Time course of tubular superoxide generation in response to anti-PR3 challenge. TNF-primed TEC were incubated with murine monoclonal anti-PR3 Abs (anti-PR3; 2.5 μg/ml and 250 ng/ml) or isotype-matched control mouse IgG (IgG; 2.5 μg/ml) for various time periods, or sham incubation was performed (control). O₂⁻ generation, as assessed by the superoxide dismutase-inhibitable reduction of ferricytochrome c, is expressed as nanomoles of O₂⁻ per milliliter of assay volume. Means ± SEM of four independent experiments each are given. *, Values differ significantly from controls (p < 0.05).

FIGURE 6. Time course of prostanoid release in response to anti-PR3 treatment. After 2 h of TNF priming, TEC were incubated with murine monoclonal anti-PR3 Abs (anti-PR3; 2.5 μg/ml and 250 ng/ml) or isotype-matched control mouse IgG (IgG; 2.5 μg/ml) in the presence of exogenous AA (5 μM). In addition, sham incubation (control) or monostimulation with AA was performed. At indicated time points, the release of PGE₂ (A) and TxB₂ (B) into the cell supernatant was quantified. Means ± SEM of six independent experiments are given. *, Values differ significantly from controls (p < 0.05).
PR3-mRNA expression correlated with crescent formation in WG patients in these studies. Importantly, in the studies of Schwarting et al. (28), proximal TEC were found to be negative for PR3 message, thus well corresponding to previous findings by King et al. (42). Together with these preceding investigations, the current immunofluorescence data clearly demonstrate that TNF exposure results in surface expression of the ANCA target Ag PR3 on distal TEC, thereby becoming accessible to PR3-ANCA. Moreover, in the present flow cytometry studies, four different monoclonal anti-PR3 Abs, recognizing different epitopes of the c-ANCA target Ag (38), bound specifically to unfixed TEC, and the immunoprecipitation experiments confirmed that the anti-PR3 Abs indeed recognized a 29-kDa protein on the tubular cells, thus leaving no doubt that PR3 is present on the surface of TNF-treated TEC.

In response to anti-PR3, but not to control Ig exposure, phosphoinositide hydrolysis with the appearance of impressive quantities of inositolphosphates was noted in the TNF-primed TEC. Notably, the magnitude of this signaling response approached that induced by bradykinin, one of the most potent activators of phosphoinositide hydrolysis in distal TEC hitherto described (46). Analysis of the kinetic response showed rapid responsiveness to the Ab binding, with maximum IP₃ accumulation appearing within 15 min.

In addition to phosphoinositide hydrolysis, a time- and dose-dependent release of superoxide anion into the supernatant of anti-PR3-treated TEC was noted. The time course of this event with maximal O₂⁻ liberation appearing 30 min after anti-PR3 admixture...
suggests induction of the superoxide formation via the phosphoinositide signaling pathway. Such suggestion is in line with the well-established sequence of phosphoinositide-derived diacylglycerol formation evoking NADPH-dependent O$_2^\bullet^-$ formation in leukocytes (47, 48). The presence of this membrane-bound multienzyme complex has previously been established in epithelia of different origin including glomerular epithelial cells and TECs (49, 50). Similarly, renal TEC O$_2^\bullet^-$ formation becomes evident only in the presence of free exogenous fatty acid AA. This observation of exogenous AA-dependent anti-PR3-elicited prostanoid generation thus suggests stimulation of the tubular COX pathway(s) under the conditions of Ab binding, with the underlying signaling steps deserving further elucidation.

A prominent increase in intracellular cAMP levels was noted in the anti-PR3-stimulated TEC. Because PGE$_2$, the predominant AA metabolite elicited by this Ab challenge, is a potent activator of renal tubular epithelial cells in vitro. This view is supported by the findings that 1) the kinetics of prostanoid formation precede those of cAMP appearance, 2) similar to the prostanoid generation itself, the anti-PR3-induced cAMP elevation is exogenous AA dependent, and 3) both prostanoid generation and cAMP accumulation are suppressed by indomethacin. The link between anti-PR3 binding and triggering of the phosphoinositide hydrolysis pathway is less obvious. Nonspecific complement-dependent activation of the TECs may be ruled out, as all experiments were performed under serum-free conditions. As unprimed TEC, lacking PR3 surface expression, were not activated by anti-PR3 treatment, specific targeting of PR3 is a prerequisite for ANCA-related activation of these epithelial cells. Importantly, isotype-matched control IgG, as compared with monoclonal anti-PR3 in all currently used assays, was not capable of eliciting renal tubular signaling and metabolic events, thus clearly demonstrating that sole ligation of Fc IgGRs is not sufficient for inducing tubular activation. Activation of cellular signal transduction pathways by anti-PR3 Abs has been described in neutrophils (60), as well as in endothelial cells (30), with activation of protein kinase C and tyrosine phosphorylation of numerous proteins being involved in the activation of the neutrophil respiratory burst by these autoantibodies (61). Clearly, the molecular mechanisms triggering TEC activation by anti-PR3 Abs deserve further investigation.

Although the initiating pathogenetic mechanisms involved in the autoimmune process in WG remain to be elucidated, the presently described ANCA-induced activation of TEC may amplify the inflammatory process once the autoantibodies have developed. Proinflammatory cytokines such as TNF-α, demanded for induction of PR3 surface expression, are well known to be elevated, both systemically and locally, at inflammatory sites in ANCA-associated renal injury (62, 63). Once the autoantigen PR3 is expressed, circulating ANCA may gain access to TEC from the basolateral side through vasculitic lesions found not only around glomeruli but also in the tubulo-interstitial compartment (3, 4, 64). Alternatively, in progressive states of renal failure, ANCA-IgG may gain access to tubules from the luminal side due to proteinuria. Reactive oxygen species arising from ANCA-TEC interaction have been implicated in a variety of renal injury mechanisms, including anti-myeloperoxidase-associated glomerulonephritis (35, 65–67), and the vasoactive lipid mediators may severely interfere with the regulation of glomerular and tubular hemodynamics (31–33) and may directly affect tubular transport functions (68, 69).

In conclusion, this study identifies distal human TECs as direct target cells for anti-PR3 Abs once the autoantigen is expressed under inflammatory conditions. The interaction of ANCA and TEC results in pronounced activation of the phosphoinositide hydrolysis-related signal transduction pathway and associated metabolic events in these epithelial cells. Arising reactive oxygen species and vasoactive prostanoids may contribute to pathophysiologic events underlying kidney injury in WG.

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References


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