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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 cytoplasmic 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 superoxide 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 cytoplasmic 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 cytoplasmic 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.
(30) and activation of signaling responses such as phosphoinositi
tide hydrolysis with progressive loss of endothelial barrier properti
(30), the effects of c-ANCA interaction with renal TECs are largely unknown. Such interaction may, however, be of major inter
est, as renal TEC may well be involved in inflammatory events, by expression of adhesion molecules, promotion of leukocyte recrui
tement, and synthesis of inflammatory mediators (6-10). Tu
bular-derived lipid mediators such as PGs and thromboxane (TX)
can cause severe vasoregulatory disturbances, thereby contribut
ing to the impairment of renal function (31-33). Moreover, TEC can pro
duce 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 epit
ethelium 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-PR3 Abs purified from WG sera
(PR3-ANCA) on signaling events and inflammatory mediator gener
ation in human renal TECs in vitro. In essence, marked stimu
lation 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 Bieist 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), 100 000 U/ml penicillin, 100 μg/ml streptomycin, and 17 U/ml heparin
(Seromed). The tissue was decapsulated, the cortex was 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) the tissue
was digested, washed twice, and finally resuspended in M199 containing
10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml t-
glutamin, 17 U/ml heparin, and 20 μg/ml endothelial cell growth factor
(Boehringer Mannheim, Mannheim, Germany). Cells were plated into 25-
cm² gelatin-coated culture flasks (Costar, Bodenheim, Germany) and
grown at 37°C with 5% CO2. After growth of 7 days they 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 18 (DAKO, Hamburg, Germany). To further determine the local
ization within the nephron TEC derived from, distal TEC were identified by
positive staining for human milk fat globulin (HMFGf, and HMFG2; Couter Immunotech, Hamburg, Germany) and GST-α (DAKO, Hamburg,
Germany), whereas Abs to GST-α (Biotrin, Dublin, Ireland), and γ-glutamyl transferase (mAb 102 D2, K2, B1, kindly provided by Dr. N. Sabo
lovic, Nancy, France) were used to identify proximal TEC. Contamination with endothelial cells (factor VIII-related Ag), monocytes (CD45, CD14),
and fibroblasts (SB5S) was excluded.

The relative percentage of proximal and distal TECs was determined by
FACS using mAb 102 D2, K2, and B1, and Abs to HMFGf, HMFG2,
GST-α, and GST-α according to standard methods. FACS analysis was per
formed 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 FACS
analysis (data not shown).
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 trichloracetic acid (v/v; final concentration, 7.5%), kept on ice for 15 min, and extracted four times with diethyl ether. 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\(_2\)), 0.1 M formic acid/0.5 M ammonium formate (for [\(^{3}H\)]IP\(_3\)), and 0.1 M formic acid/1 M ammonium formate (for [\(^{3}H\)]IP\(_4\)). 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 TECs 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 quantification 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 stream of nitrogen and stored at −70°C.

Release of PGE\(_2\) and TxB\(_2\), the stable metabolite of TxA\(_2\), into the cell supernatant was quantified 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 Lund, Sweden), whereas the anti-PR3 Ab 12.8 was from Research Diagnostics (Flanders, NY). 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\(_3\), IP\(_2\), and IP\(_1\), collectively depicted as IP\(_x\), 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\(_x\) 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 \( \mu \)g/ml), whereas corresponding amounts of murine and human control IgG turned out to be completely ineffective.

\( \text{O}_2^\text{\textsuperscript{\text{-}}} \) generation

The anti-PR3-evoked signaling response was accompanied by marked \( \text{O}_2^\text{\textsuperscript{\text{-}}} \) formation (Fig. 5). In TNF-primed TEC, anti-PR3 challenge provoked a time- and dose-dependent secretion of \( \text{O}_2^\text{\textsuperscript{\text{-}}} \), 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 \( \mu \)g/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 \( \text{O}_2^\text{\textsuperscript{\text{-}}} \).

Lipid mediator release

Being a characteristic metabolic property of distal TECs, the generation of the cyclooxygenase (COX) metabolites of AA, PGE\textsubscript{2}, and TxA\textsubscript{2} 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 \( \mu \)M) resulted in a prominent release of PGE\textsubscript{2} and, to a lesser extent, TxB\textsubscript{2}, the stable metabolite of TxA\textsubscript{2}, into the cell supernatant of anti-PR3-stimulated cells (Fig. 6). Application of sole AA resulted in a 2-fold increase, while 2.5 \( \mu \)g/ml anti-PR3 plus AA elicited an -6-fold increase in PGE\textsubscript{2} and TxB\textsubscript{2} over controls. Lipid mediator release peaked after 30 min of incubation, with 2.5 \( \mu \)g/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 \( \mu \)M) (Fig. 7).

Intracellular cAMP levels

Because PGE\textsubscript{2}, the predominant renal tubular COX product elicited by anti-PR3 challenge, is a potent activator of adenylateclase, 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 PGE\textsubscript{2} (Fig. 8). As anticipated from the data in PGE\textsubscript{2} 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-\( \alpha \). 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).
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$_2^-$ 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). Alternatively, renal TEC O$_2^-$ generation might occur via xanthine oxidase (51) and NO synthetase (52), both calcium-dependent enzymes, which may respond to IP$_3$-mediated release of this bivalent cation from intracellular stores.

Similar to the superoxide generation, tubular epithelial prostano- noid synthesis peaked after 30 min, again suggesting secondary triggering via the preceding phosphoinositide hydrolysis-related signaling cascade. The phosphoinositide pathway is well known to be linked with phospholipolytic activities, resulting in AA liberation from various intracellular membrane pools and thereby triggering prostanooid generation via the COX pathway (53, 54). However, the currently observed generation of PGE$_2$ and Tx in the anti-PR3-challenged TEC demanded the exogenous supply with the precursor fatty acid AA. This finding corresponds to observations in granulocytes, macrophages, and alveolar type II cells, in which, in response to receptor-operated stimuli, AA metabolite formation becomes evident only in the presence of free exogenous AA (55, 56). Under inflammatory conditions, extracellular AA is assumed to be readily available, estimated to range >10 μM in an inflammatory focus, i.e., above the concentration presently provided (5 μM) to the renal epithelial cells (57, 58). The current observation of exogenous AA-dependent anti-PR3-elicted prostano- noid 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 adenylatecyclase (53, 59), it may well be responsible for the renal tubular CAMP elevation. This view is supported by the findings that 1) the kinetics of prostanooid formation precede those of c-AMP appearance, 2) similar to the prostanooid generation itself, the anti-PR3-induced CAMP elevation is exogenous AA dependent, and 3) both prostanooid 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 present description of 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 prostanooids may contribute to pathophysiologic events underlying kidney injury in WG.

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of the antigens involved, the assays, and the clinical and possible pathogenic consequences. Blood. 1996. 1.


