Human renal tubular epithelial cells as target cells for antibodies to proteinase 3 (c-ANCA)

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Introduction

Circulating autoantibodies directed against neutrophil cytoplasmic antigens (ANCA), especially those with specificity for proteinase 3 (PR-3) are specific markers for systemic vasculitides such as Wegener's granulomatosis (WG) and microscopic polyarteritis. Several in vitro studies support the hypothesis that ANCA are more than an epiphenomenon and are intimately involved in the pathogenesis of these disorders. Recently, we were able to demonstrate a direct effect of anti-PR3-ab on neutrophil-endothelial cell interaction [1]. Binding of anti-PR3-ab to their antigen translocated into the membrane of human endothelial cells (HEC) leads to both enhanced adhesion of neutrophils via induction of E-selectin and enhanced adhesion of T-lymphocytes to HEC via expression of VCAM-1 [2]. Lesions in renal biopsies from patients with WG are characterised by necrotizing, crescentic pauci-immune glomerulonephritis (GN) and a marked influx of leukocytes both within glomeruli and interstitium [3–5]. Accumulation of cells in the extracapillary space of glomeruli and tubuli includes monocytes/macrophages, epithelial cells and T-lymphocytes [6–8]. Previous studies have shown that tubular epithelial cells (TEC) seem to play an active role in the tubulo-interstitial injury by producing proinflammatory cytokines (PDGF, GMCSF, IL-6, TNF) [9–11] and expressing MHC II molecules [12,13] and adhesion molecules like ICAM-1 and VCAM-1 [14–16]. Brouwer et al. recently found the lysosomal enzymes PR-3, myeloperoxidase (MPO) and human leukocyte elastase (HLE) in tubular casts and within TEC in renal biopsies of patients with WG [5]. Further immunohistologic studies showed that PR-3 is not only located on crescent glomeruli but also on the tubulo-interstitium [17].

Thus, it was hypothesised that interaction of ANCA with TEC could contribute to renal lesions associated with WG. The aim of our study was to investigate the effect of anti-PR-3 ab binding to TEC. Initially, we have studied if PR-3 can be expressed by TEC at the mRNA-level. Secondly, we have investigated the effect of anti-PR-3 ab on the expression of the adhesion molecules ICAM-1 and VCAM-1 by TEC.

Patients and methods

Serum samples

Serum samples were obtained from 185 donors. 50 suffered from clinically active WG. The diagnosis was established on the basis of classical symptoms and the typical histological findings in biopsy specimens according to the criteria of the American College of Rheumatology [18] and the Chapel Hill Consensus Conference [19].

Antibody testing

All WG-sera were tested for anti-PR-3 ab by IFT on ethanol fixed neutrophils and enzyme-linked immunosorbent assay (ELISA). Several antigen preparations served as antigens: α-extract of human neutrophils, purified PR-3, myeloperoxidase, cathepsin G and HLE. ELISAs were performed as described earlier [20]. Polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed as described previously [21].

In addition, all sera were screened for antibodies to endothelial and tubular epithelial cells by immunofluorescence. Rheumatoid factor (RF) was determined by a latex fixation test (Behring Werke Marburg, Germany).

Purification of anti-PR-3 antibodies

IgG was prepared from 5 anti-PR-3 ab positive WG-sera (c-ANCA titres 1:80–1:640) by ammonium sulfate precipitation and ion exchange chromatography on DEAE-SEphadex (Pharmacia, Uppsala, Sweden). Anti-PR-3 ab were affinity-purified as described earlier [21] using purified PR-3.

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**Preparation of cell-extracts**

An extract (x-fraction) of human neutrophils was prepared as described by Rasmussen et al. [22] and Savage et al. (‘acid’ extract) [23]. Only cell cultures free of fibroblasts or monocytes obtained after several passages were used for these experiments.

**Purification of PR-3**

PR-3 was purified as described by Kao et al. [24] and affinity purified as described by Lüdemann et al. [25] using an extract of granulocytes. For inhibition experiments, serum antibodies were mixed with purified PR-3 vol/vol diluted to 0.1 mg/ml protein concentration in phosphate-buffered saline (PBS), and incubated on a rotator for 1 h at 37°C and 12 h at 4°C. The mixture was centrifuged at 30000 g for 15 min at 4°C and the supernatants kept as absorbed material.

**Isolation and culture of renal tubular epithelial cells**

TEC were isolated from human kidney portions obtained from the normal pole of kidneys removed for renal carcinoma as described by Van Der Biest et al. [26]. The macroscopically normal tissue was dissected and transferred into ice cold medium M199 (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco, Eggenstein, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin, 17 units/ml heparin, L-glutamine (Seromed). The tissue was decapsulated, the cortex discarded and the outer medulla was sectioned into pieces of 1 mm³. The tissue was incubated in Hank’s balanced salt solution (HBSS) containing 0.2% collagenase II (Sigma, St. Louis, USA) for 1 h at 37°C with gentle shaking. The suspension was filtered through a 120 μm sieve. The cells were washed twice and resuspended in 20 ml M199. The resulting suspension was centrifuged with a Metrizamide gradient (Nycoderm, Oslo). All material from the top of the gradient were collected, since staining of that fraction revealed, that the majority of the TEC is confined to this fraction. Cells were washed twice with HBSS, finally resuspended in M199 containing 20% FCS, 50 μg/ml Heparin (Hoffmann La-Roche, Grenzach, Germany) and 20 μg/ml ECGF (Boehringer Mannheim, Germany) and plated into 75 mm² culture flasks (Greiner, Frickenhausen, Germany).

**Identification of TEC**

The cells were grown to confluence and passaged with a split ratio of 1:3. After the first passage the cells revealed a homogeneous morphology. The epithelial origin of these cells was confirmed by immunohistochemical staining. Cultured TEC were characterised using abs to cytokeratin 8, cytokeratin 18 (Dianova, Hamburg, Germany), human milk fat globulin (HMFG) number 1 and number 2 (Immunotech, Hamburg, Germany), epithelial membrane antigen (EMA) (Dako, Glostrup, Denmark), γ glutamyl-transferase (GGT) (MoAb 102 D2, B1, kindly provided by Dr Sabolovic, Nancy, France), neutral endopeptidase (CD 10, Dako), villin (Serotec, Oxford, UK), glutathione-s-transferase z (GST-z) and glutathione-s-transferase π (GST-π) (Biotrin, Dublin, Ireland), vimentin, CD14, 5B5 and factor VIII-related antigen (Dianova, Hamburg, Germany). The relative percentage of proximal (PTEC) and distal (DTEC) tubular epithelial cells was determined by fluorescence activated cell sorting (FACS) using MoAb D1k, B1, and abs to HMFG1, HMFG2 and GST-z and GST-π. FACS analysis was performed in a FACScan (Becton-Dickison, Mountain View, CA, USA) using forward and orthogonal light scatter to select viable cells. Data for 3000 cells were collected.

**Immunofluorescence studies**

TEC were passaged on gelatine-coated 8-well chamber slides (Lab-Tek, Miles-Scientific, Naperville, IL, USA) and incubated in a humidified atmosphere with 5% CO2 and 37°C for 24 h. Confluent cells were stimulated with 10 μg/ml purified anti-PR-3 IgG in the presence or absence of purified PR-3 (as described above) or 10 μg/ml normal human IgG (Sigma, Deisenhofen, Germany). Unfixed cells were washed twice with PBS and incubated with the second antibody, fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (F-5512; Sigma, Germany). Stained cells were analysed with a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany).

**Binding of anti-PR-3 ab to TEC**

**Cell-based ELISA**. Binding of anti-PR-3 ab to TEC was determined by Cyto-ELISA technique as described by Frampton et al. [27] with minor modifications. Cyto-ELISAs were carried out with TEC grown to confluence on 0.1% gelatine coated 96 well plates in M199–10% FCS. Plates were incubated as described above. At different times (0, 1, 2, 4, 8 and 12 h) cells were incubated with purified IgG anti-PR-3 (abs 10 μg/ml) or normal human IgG (10 μg/ml) in the presence or absence of cytokines (IL-1 β 25 U/ml, IFN-γ 400 U/ml, TNF-α 4 ng/ml, TGF-β 1 ng/ml) (Boehringer Ingelheim, Germany) (Figure 5a and b). To block unspecific binding to Fc γ receptors experiments were performed with TEC preincubated with 1 mg/ml heat-aggregated human IgG as described by Rekvig and Hannestad [28]. Culture medium was removed, the adherent, unfixed cells were washed with phosphate buffer solution (PBS) (Gibco, Eggenstein, Germany), then blocked for 1 h in PBS/2% bovine serum albumine (BSA) (Seromed). The tissue was sectioned into pieces of 1 mm³. The suspension was centrifuged at 30000 g at 4°C, and the supernatants kept as absorbed material. The mixture was centrifuged into a Metrizamide gradient (Nycomed, Oslo). All material from the top of the gradient were collected, since staining of that fraction revealed, that the majority of the TEC is confined to this fraction. Cells were washed twice with HBSS, finally resuspended in M199 containing 20% FCS, 50 μg/ml Heparin (Hoffmann La-Roche, Grenzach, Germany) and 20 μg/ml ECGF (Boehringer Mannheim, Germany) and plated into 75 mm² culture flasks (Greiner, Frickenhausen, Germany).

**Expression of VCAM-1 and ICAM-1 on TEC**

**Cell-based ELISA**. The expression of adhesion molecules on cultured, unfixed TEC was determined by Cyto-ELISA-technique as described above. At different times (Figure 6a and b) cells were incubated either with 10 μg/ml purified IgG anti-PR-3 abs, 10 μg/ml normal human IgG or with IFN-γ(400 U/ml) or TNF-α (4 ng/ml). Culture medium was removed, the adherent cells were washed and incubated with a 1:100 dilution of the second antibody, a peroxidase (POD)-conjugated anti-human IgG (Sigma) for 1 h and washed again. The activity was determined as optical density (OD) at 492 nm. The assays were carried out in triplicate. Similar assays were performed using cultured TEC from four different patients.
Detection of PR-3 mRNA in TEC

Detection of PR-3 mRNA was performed by reverse transcription polymerase chain reaction (RT-PCR) method. Total cellular RNA was isolated using a guanidinium thiocyanate method [29]. RNA was isolated from unstimulated TEC at passage 3–4, from TEC after stimulation with IL-1β (25 U/ml) and from HL-60 cells, a myelomonocytic cell line (positive control). 1 µg HL-60 RNA, 1 µg TEC RNA (unstimulated) and 1 µg TEC RNA (IL-1-1–stimulated) were subjected to reverse transcription. RNA was linearised by 5 min heating at 80°C and 1 min cooling on ice. RT reagent mixture was added and RT performed for 1 h at 37°C using the ‘random priming’ method. RT reagent mixture consisted of 1 µl 20 µM random hexamer primers, 1 µl dNTP mix (10 mM each), 4 µl 5 × reaction mix (final concentration: 50 mM Tris-HCl, pH 8.3, 75 mM KCL and 3 mM MgCl2), 0.5 µl RNAse inhibitor (50 U/µl), 1 µl M-MLV reverse transcriptase (recombinant Moloney-Murine Leukemia Virus reverse transcriptase, 200 U/µl) (Stratagene, Heidelberg, Germany) and DEPC-treated water up to 20 µl total volume. This mixture was incubated at 37°C for 1 h. The reaction was terminated by heating to 80°C for 10 min and quickly chilling in ice. Five to 10 µl were removed for PCR amplification. Amplification was started with 10 µl transcribed material and 40 µl PCR mixture. PCR mixture consisted of buffer, 50 pmol sense and anti-sense primers, 0.1 mM dNucleotides and 1.25 U Taq polymerase (Stratagene). The 50 µl reactions were covered with mineral oil and incubated in a Perkin Elmer Cetus thermal cycler using 30 cycles of 1 min 95°C and 2 min 72°C primer annealing temperature and polymerase extension temperature. Ten µl of each RT-PCR reaction were electrophoresed on 1% agarose gels. The following primers were used in this study:

PR-3 ‘sense’: 5’ATCGTGGGCCGGCAAGGAGCG (at the beginning of exon 2, corresponding to bases +553 to +101 of the cDNA)
PR-3 anti-sense: 5’CGGGGCGGGAGAAAGTGCA (at the end of exon 4, corresponding to bases +553 to +582)

Results

Characterisation of cultured renal tubular epithelial cells

Cultured TEC were characterised by immunofluorescence and FACS-analysis. Since cells can loose characteristics of antigen expression under culture conditions we have used different antibodies with proven specificity for the differentiation of TEC even after in vitro culture [26]. TEC isolated and cultured under our conditions showed a strong staining for cytokeratin 8,-18, EMA, HMFG1, HMFG2 and GST-π (Figure 1). No staining or only a weak staining was obtained using abs to villin, GGT, GST-α or CD 10 (Figure 1). After the first passage also no contaminating fibroblast (5B5), endothelial (F VIII Ag) or monocytic (CD 14) cells were detected (not shown). According to Van der Biest and co-worker this staining pattern is typical for TEC from the distal part of the nephrons [26]. The anti-HMFG MoAbs specifically recognize cells of the thick ascending limb, the distal convoluted tubule and the collecting duct [30]. The expression of EMA and GST-π is preferentially restricted to distal nephron segments [31,32], whereas the brush border enzyme GGT is strongly expressed in confluent PTEC monolayers [26,33]. The GST comprise a multigene family of detoxification enzymes, which are structurally and functionally distinct [34]. GST-α, formerly known as ligandin, is present in the proximal convoluted tubules in rat and man [35]. Due to the distinct distribution of GST-α and GST-π these GST-isoenzymes have also been used as markers for tubular necrosis or acute rejection after kidney transplantation [36]. CD 10 (neutral endopeptidase) is found in abundant amounts in the brush border of PTEC and can also be detected under culture conditions [37].

The relative percentage of PTEC and DTEC in our cultured TEC was further evaluated by FACS-analysis. Representative experiments are shown in Figure 2. The majority of cultured TEC were characterized as DTEC under our culture conditions varying from 92–97% anti-HMFG and anti-GST-π positive cells in contrast to 11–17% anti-GGT and anti-GST-α positive TEC. Thus, FACS-analysis confirmed the results of immunofluorescence studies. TEC from different patients (n = 4) did not significantly influence the relative percentage of DTEC or PTEC (data not shown).

Detection of PR-3 mRNA in TEC

Using random priming RT-PCR we could detect a 500 bp fragment of PR-3 in unstimulated and IL-1β (25 U/ml) stimulated TEC (Figure 4). PR-3 cDNA from HL-60 cells (Lane 1), a myelo-monocytic
cell-line (as positive control), and TEC (Lane 2: 1 µg, unstimulated; Lane 3: 1 µg, IL-1-stimulated) were amplified by RT-PCR. Lane 4 contains the negative control (H₂O) and Lane 6 contains Hinc II × 174 digests (USB-Amersham, Braunschweig) as size markers. The identity of the PCR-product was verified by nucleotide sequencing after cloning in bluescript ks+ (Stratagene) according to standard methods [38].

Specificity of antibodies and binding to TEC

Five out of 50 selected WG-sera were positive for anti-PR-3 ab as determined by IFT on human neutrophils (C-ANCA +; titres 1:80–1:640), by ELISAs with z-fraction and ‘acid’ extract of human neutrophils by Western blot (reaction at 29 kDa) and had no anti-endothelial cell activity on unstimulated HUVEC. Ab reactivity could be blocked by incubation with purified PR-3 antigen as measured by ELISA and determined by Western blot [1].

Purified IgG anti-PR-3 abs produced a strong diffuse cytoplasmic staining of TEC (Figure 3c) in comparison

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Fig. 2. Representative FACS-analysis of cultured TEC using monoclonal Abs to (A) GST-pi (97% positive), (B) HMFG₁/HMFG₂ (96% positive), (C) GST-α (17% positive) and (D) GGT (11% positive).

Fig. 3. (A) Indirect immunofluorescence of unfixed TEC (no pretreatment with anti-PR-3 ab; original magnification ×450). No reaction with the anti-human IgG FITC-conjugated second ab. (B) Binding of human normal IgG to unfixed TEC with a weak diffuse background staining. (C) Reaction of a purified anti-PR-3 ab on TEC with diffuse cytoplasmic staining (original magnification ×450).
to the negative control (treatment only with second antibody on cultured TEC) (Figure 3a) and the binding of human control IgG (Figure 3b).

Cytokine treatment of TEC led to a time dependent modulation of anti-PR-3 ab binding to TEC. Figure 5 shows an increased surface expression of PR-3 under the influence of TNF-α and IL-1β on unfixed TEC with a maximum after 8 h, whereas TGF-β down regulates the expression of PR-3. IFN-γ had no further effect on the binding capacity of TEC towards anti-PR-3 ab. Assays were performed three times and values given are the mean of three measurements. Data including mean ± S.D. are given in Table 1.

Expression of adhesion molecules VCAM-1 and ICAM-1 on TEC

ICAM-1 and VCAM-1 were constitutively expressed on TEC under culture conditions (Figure 6a and b). Incubation of TEC with purified ab to PR-3 led to a marked increase of VCAM-1 expression with a peak after 4 h compared to the effects obtained with control human IgG (P < 0.001) (Table 1). There was only a slight, but significant increase of ICAM-1 expression at 2 h after incubation with purified ab to PR-3 (P < 0.05) (Table 1). Representative examples of the time course of VCAM-1 and ICAM-1 expression in response to anti-PR-3 abs are presented in Figure 6.

To further investigate the biological relevance of the effects observed we have compared the antibody mediated effects with effects induced by proinflammatory cytokines. Incubation of TEC with IFN-γ led to the highest expression of ICAM-1 with a peak after 2 h. The effect of TNF-α on ICAM-1 expression was similar to the effect observed after incubation with anti-PR-3 abs. IFN-γ and anti-PR-3 abs showed similar levels of VCAM-1 expression on TEC but different kinetics with a peak at 24 h after incubation with IFN-γ (Figure 6a and b). There was only a slight increase in VCAM-1 expression after incubation with TNF-α. The baseline values given in Figure 6a and b representing incubation with normal human IgG are the mean of three measurements at each time (0, 1, 2, 4, 8 and 12 h), that is at six different time points. The binding of isotype-matched, non-specific mouse immunoglobulins incubated with purified ab to PR-3 abs or normal human IgG in the presence or absence of cytokines (IL-1β, IFN-γ, TNF-α, TGF-β) are given in Table 1. One mark represents the mean of three measurements (data including mean ± S.D. are given in Table 1).

Discussion

The primary function of TEC, the most abundant cell type in the kidney cortex is solute and water transport. Increasing evidence supports the concept that TEC act as immune accessory cells since they can be induced to express MHC II antigens [39,40] and adhesion molecules [43,44] like ICAM-1 and VCAM-1, as well as to secrete cytokines [9–11] and process potentially immunogenic peptides from blood and the glomerular filtrate [41,44].

Until now several concepts concerning a direct role of anti-PR-3 ab in the pathogenesis of WG have been discussed in literature. Brouwer et al. [5] have shown
that PR-3, MPO and HLE were also located in tubular casts and within TEC. They supposed that these lysosomal enzymes were taken up by TEC as cationic proteins. In the present study we could now demonstrate for the first time that TEC can express PR-3 mRNA under the influence of proinflammatory cytokines. Binding of purified anti-PR-3 ab to TEC led to a marked increase of adhesion molecule expression suggesting that PR-3 is translocated to cell surface. There, PR-3 becomes accessible as target antigen for anti-PR-3 ab. Similar effects of anti-PR-3 ab have been observed on neutrophils and endothelial cells. Falk et al. were the first to demonstrate that ANCA can activate neutrophils to produce reactive oxygen species and release lysosomal enzymes [45]. Pre-treatment ('priming') of neutrophils with cytokines like TNF-α, however, is necessary for the expression of lysosomal enzymes such as PR-3 in the cell membrane [46].

Binding of anti-PR-3 ab to primed neutrophils results in further activation. In addition, our group could recently detect PR-3 in HUVEC. PR-3 is also translocated to the cell membrane under the influence of cytokines like TNF-α and IL-1 β [1]. Furthermore, we could demonstrate that binding of anti-PR-3 ab on HUVEC led to a direct cytotoxic effect on vascular endothelium [2] or to an enhanced adhesion of neutrophils, monocytes and lymphocytes via the induction of E-selectin and VCAM-1 [47,48].

The interaction of ANCA with HUVEC closely resembles the effects of ANCA on TEC. In the present study we could show that VCAM-1 and ICAM-1 expression on TEC are upregulated in vitro after stimulation with anti-PR-3 ab (Figure 6 a and b). However, direct immunofluorescence studies have only revealed minimal amounts of immune deposits in renal lesions of ANCA-related glomerulonephritis (‘pauci-immune’). This could be explained by the finding of Dolman et al., who described proteolysis of c-ANCA IgG complexed to the enzyme.

VCAM-1 and its ligand VLA-4 play an important role in the in vitro adherence of monocytes and activated T-cells to endothelium [50]. VCAM-1, however, is expressed not only by cytokine activated endothelium in the kidney, but also by non-vascular cells such as TEC and mesangial cells [16]. Previous studies have shown that VCAM-1 expression on TEC can be upregulated by addition of cytokines like TNF-α, IFN-γ and IL-1 β [16]. VCAM-1 expression on TEC induced by inflammatory cytokines is functionally capable of binding VLA-4 on lymphoid cells [51].

In vasculitis, glomerular as well as tubular epithelial VCAM-1 expression was found [52]. VCAM-1 expression on TEC was most marked in biopsies of patients with interstitial nephritis or systemic vasculitis with crescent nephritis [52]. One characteristic finding in WG is the accumulation of monocytes, leukocytes and T-cells in the tubulo-interstitium. Since our results demonstrate that even anti-PR-3 ab are able to induce VCAM-1 expression on TEC, this might be the initial step for monocyte- or T-cell migration to the tubulo-interstitial tissue.

Upregulated ICAM-1 expression has also been found in TEC with extracapillary GN [53]. In contrast to VCAM-1 the intercellular adhesion molecule ICAM-1 is expressed in a variety of tissues. In addition to the vascular presence of ICAM-1, a common finding in normal kidneys, expression of ICAM-1 on TEC was observed in normal and diseased tubules in GN [15].

Similar to endothelial cells, which form the barrier between the circulation and the surrounding tissue, renal tubule epithelia are localised between internal and external milieu, regulating internal homeostasis. In all forms of progressive GN a major tubulo-interstitial infiltrate of immune-competent cells is present. All recent studies of the outcome of different forms of progressive GN concur that the presence and severity of tubulo-interstitial changes is a major factor determining outcome [54]. Our data demonstrate that ANCA are able to modulate the interaction between tubule epithelia and monocytes, leukocytes and T-cells. Thus, TEC could even initiate immune reactions in ANCA related vasculitides. Even though our cultured TEC were a mixture of proximal and distal TEC the majority of cells studied derived from distal nephron.
segments indicating that not only proximal but also distal TEC may act as immune accessory cells.

In summary, our data support the hypothesis, that anti-PR-3 ab in interaction with immune accessory TEC may play an important role in the pathogenesis of ANCA related GN.

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