Activation of podocytes by mesangial-derived TNF-α: glomerulo-podocytic communication in IgA nephropathy


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Submitted 10 September 2007; accepted in final form 29 January 2008

Lai KN, Leung JC, Chan LY, Saleem MA, Mathieson PW, Lai FM, Tang SC. Activation of podocytes by mesangial-derived TNF-α: glomerulo-podocytic communication in IgA nephropathy. Am J Physiol Renal Physiol 294: F945–F955, 2008. First published February 6, 2008; doi:10.1152/ajprenal.00423.2007.—We have previously documented that human mesangial cell (HMC)-derived TNF-α is an important mediator involved in the glomerulo-tubular communication in the development of interstitial damage in IgA nephropathy (IgAN). With the strategic position of podocytes, we further examined the role of mesangial cells in the activation of podocytes in IgAN. There was no binding of IgA from patients with IgAN to podocytes. Podocytes cultured with IgA from patients with IgAN did not induce the release of growth factors or cytokines. Furthermore, podocytes did not express mRNA of known IgA receptors. In contrast, IgA-conditioned medium (IgA-HMC medium) prepared by culturing HMC with IgA from patients with IgAN for 48 h significantly increased the gene expression and protein synthesis of TNF-α by podocytes with a 17-fold concentration above that of IgA-HMC medium. The upregulation of TNF-α expression by podocyte was only abolished by a neutralizing antibody against TNF-α but not by other antibodies. Exogenous TNF-α upregulated the synthesis of TNF-α by podocytes in an autocrine fashion. IgA-HMC medium prepared with IgA from patients with IgAN also significantly upregulated the expression of both TNF-α receptor 1 and 2 in podocytes. Our in vitro finding suggests podocytes may play a contributory role in the development of interstitial damage in IgAN by amplifying the activation of tubular epithelial cells with enhanced TNF-α synthesis after inflammatory changes of HMC.

tumor necrosis factor-α; mesangial cell; tubulointerstitial injury

GLOMERULAR PODOCYTES ARE HIGHLY DIFFERENTIATED CELLS THAT PLAY A KEY ROLE IN MAINTAINING THE INTEGRITY OF THE GLOMERULAR FILTRATION BARRIER. IN THE MATURE GLOMERULUS, PODOCYTES HAVE A LOW LEVEL OF DNA SYNTHESIS AND DO NOT RAPIDLY PROLIFERATE UNDER NORMAL CONDITIONS OR IN VARIOUS RENAL DISEASES, LIKE THE CONSEQUENCE OF A ROBUST EXPRESSION OR UPREGULATION OF CYCLIN-DEPENDENT KINASE INHIBITORS. AN INABILITY TO REPOPULATE A DAMAGED GLOMERULUS WITH FUNCTIONAL PODOCYTES CORRESPONDS WITH THE PROGRESSIVE ULTRASTRUCTURAL LESION SEEN IN PODOCYTES DURING FILTRATION BARRIER FAILURE. IN GLOMERULAR DISEASES, PODOCYTE DAMAGE LEADS TO INCREASED GLOMERULAR BARRIER PORE SIZE, ALLOWING THE PASSAGE OF PROTEINS OR OTHER MEDiators TO THE TUBULAR LUMEN (6). THIS RESULTS IN PROTEINURIA AND PROGRESSIVE LOSS OF KIDNEY FUNCTION. WHILE RECENT STUDIES HAVE LOOKED AT CONGENITAL NEPHROSIS, IN VITRO STUDIES OF PODOCYTES IN OTHER ACQUIRED GLOMERULAR DISEASE ARE SCARCE DUE TO DIFFICULTIES IN PROPAGATING PODOCYTES IN SUFFICIENT NUMBER FOR EXPERIMENTS. NORMALY, THE GLOMERULAR BARRIER IS IMPERMEABLE TO PROTEINS.

IgA nephropathy (IgAN), the most common form of glomerulo-nephritis, is one of the leading causes of renal failure in many parts of the world. The disease is characterized by mesangial deposition of pathogenic polymeric IgA1 (pIgA1; Ref. 17), proliferation of mesangial cells, increased synthesis of the extracellular matrix, and infiltration of macrophages, monocytes, and T cells (2, 13). The severity of tubulointerstitial damage in IgAN correlates closely with the declining renal function and the long-term clinical outcome (8, 29). The absence of IgA binding through known IgA receptors and the lack of tubular epithelial cell (TEC) activation support the pathologic finding that IgA is rarely deposited in the tubulointerstitium in IgAN (3, 9). The most important and central question is how glomerular mesangial IgA deposits lead to tubulointerstitial damage resulting in end-stage renal failure. Recently, we discovered a novel mechanism involving glomerulotubular cross talk (independent of proteinuria) in the development of tubulointerstitial damage in IgAN (3). Mediators (mainly TNF-α) released from mesangial cells after IgA deposition activate TEC and lead to subsequent inflammatory changes in the renal interstitium.

Podocytes are positioned strategically along the glomerulo-tubular axis, yet their role in mediating the glomerulo-tubular cross talk in IgAN has not been addressed. In this study, we examined the expression of IgA receptors and studied any direct effect of IgA molecules on podocytes. The pathophysiological effects on podocytes by mediators released from mesangial cells triggered by IgA deposition were further investigated.

MATERIALS AND METHODS

Patients and controls. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee for Studies in Humans. All subjects (patients and healthy/disease controls) gave their written informed consent for participation. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee for Studies in Humans. All subjects (patients and healthy/disease controls) gave their written informed consent for participation. Twenty-two Chinese patients (10 male and 12 female) with clinical and renal immunopathological diagnosis of primary IgAN were studied. IgAN was diagnosed by the presence of predominant granular IgA deposits, mainly in the glomerular mesangium by immunofluorescence examination, and the presence of mesangial electron-dense deposits in ultrastructural examination. All the patients were symptomatic for >12 mo, and no significant renal impairment was documented. Systemic lupus erythematosus, Henoch-Schönlein purpura, and other connective tissue diseases, and a history of infectious mononucleosis were excluded. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee for Studies in Humans. All subjects (patients and healthy/disease controls) gave their written informed consent for participation.

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purpura, and hepatic diseases were excluded by detailed clinical history, examination, and negative laboratory findings for hypocomplementemia, anti-DNA antibody, or hepatitis B virus surface antigen. Twenty milliliters of blood were collected from each patient at clinical quiescence. The serum was isolated and frozen at \(-20^\circ\text{C}\) until isolation of IgA. Every IgA preparation was separately obtained from each individual patient or healthy control.

Fifteen healthy subjects (7 male and 8 female), comparable in age and race, with no microscopic hematuria or proteinuria, were recruited as normal controls. Another 15 patients, 10 with minimal change nephropathy and 5 with Henoch-Schönlein purpura, were also recruited as disease controls. Serum was similarly collected from these individuals for processing.

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**Table 1. Semiquantitative analysis of the mRNA expression of IgA receptors by RT-PCR (from 0 to 3+)**

<table>
<thead>
<tr>
<th>Amplicon size</th>
<th>FcRcR</th>
<th>ASGPR H1</th>
<th>ASGPR H2</th>
<th>plgR</th>
<th>TIR</th>
<th>FcR/μR</th>
<th>GAPDH</th>
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<td>Podocyte</td>
<td>—</td>
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<td>—</td>
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<td>—</td>
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<td>HT29</td>
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<td>HepG2</td>
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<td>HMC</td>
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<td>PBMC</td>
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<td>—</td>
<td>weakly+</td>
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<td>U937</td>
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<td>Negative control</td>
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PBMC, peripheral blood mononuclear cells; HMC, human mesangial cells; FcRcR, Fc-\(\alpha\) receptor; FcR/μR, Fc-\(\alpha\)/\(\mu\) chain receptor; ASGPR H1 and ASGPR H2, H1 and H2 chains of asialoglycoprotein receptor; plgR, polymeric-immunoglobulin receptor; TIR, transferrin receptor.

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**Cell culture.** Cell lines, including the colonic adenocarcinoma line (HT29), the hepatocellular line (HepG2), and the monocytic line (U937), were obtained from American Type Culture Collection (Rockville, MD). These cell lines, cultured under the recommended condition, were used as positive or negative controls in various experiments. Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll gradient separation. Isolation, characterization, and culture of human mesangial cells (HMC), proximal tubular epithelial cells (PTec), and human umbilical vein endothelial cells (HUVEC) were performed as previously described (4, 18, 24). A conditionally immortalized human podocyte cell line was established in our laboratory by transfection with a temperature-sensitive SV40-T gene (30). At the permissive temperature of \(37^\circ\text{C}\), these cells grow into cobblestone morphology. Differentiated human podocytes that are grown at \(37^\circ\text{C}\) expressed markers of differentiated podocytes in vivo including nephrin.

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**Fig. 1.** Synthesis of TNF-\(\alpha\) in human mesangial cells (HMC) and podocytes cultured with IgA. A: TNF-\(\alpha\) synthesis was not increased in podocytes cultured with IgA prepared from patients with IgA nephropathy (IgAN) when compared with minimal change nephropathy (MCNS), Henoch-Schönlein purpura (HSP), healthy controls (Ctl), or incubation with culture medium (Medium Ctl) alone. B: TNF-\(\alpha\) was upregulated when mesangial cells were cultured with polymeric IgA (plgA) prepared from IgAN patients when compared with healthy or disease controls. The concentration of TNF-\(\alpha\) in the supernatant of mesangial cells cultured with plgA prepared from healthy controls was \(115.3 \pm 18.7\) pg/ml.

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**Fig. 2.** Upregulation of TNF-\(\alpha\) mRNA and protein expression in podocytes cultured with IgA-HMC conditioned medium. Gene (A) and protein (B) expression of TNF-\(\alpha\) was upregulated in podocytes cultured with IgA-HMC conditioned medium prepared from IgAN patients (solid bars) compared with that from healthy controls (open bars). Similar findings were not observed with podocytes cultured with conditioned medium prepared from podocytes, proximal tubular epithelial cells (PTec), or human umbilical vein endothelial cells (HUVEC) incubated with the same plgA preparation. Results are means \(\pm\) SD from 6 individual experiments.
were washed with staining buffer and then further incubated with staining was achieved by reaction with the preimmune F(ab\textsuperscript{2})/H11032/H9262 concentration of 50 \(\mu\)g/ml. The cells were incubated with 100 \(\mu\)l of rabbit anti-human IgA antibody. Background control controls as described previously (19). Human mesangial cells or experiments, differentiated podocytes between passages 12 to 17 were used and were growth arrested with culture medium containing 0.5% FBS for 24 h before the commencement of experiments.

Isolation of pIgA and flow cytometry. pIgA1 (molecular mass \(>320\) kDa) was isolated and purified from sera of patients with IgAN or controls as described previously (19). Human mesangial cells or podocytes were grown to log phase and harvested by using 0.05% trypsin/0.02% EDTA for 5 min at room temperature. The cells were adjusted to \(5 \times 10^6\) per ml, and 200 \(\mu\)l of cell suspension were used in binding assays. Staining was performed at 4°C with staining buffer consisting of PBS with 1% FBS and 0.1% sodium azide. The cells were incubated with 100 \(\mu\)l of pIgA (final concentration of 50 \(\mu\)g/ml) for 30 min. After incubation, the cells were washed with staining buffer and then further incubated with 100 \(\mu\)l of rabbit anti-human IgA antibody. Background control staining was achieved by reaction with the preimmune F(ab\textsuperscript{2})\textsubscript{2} fragment of fluorescein-conjugated isotypic antibody. The stained cells were analyzed using a Coulter EPICS XL analyzer (Miami, FL). A minimum of 5,000 fixed cells for each sample was analyzed. Fluorescence intensity was evaluated by comparing the mean fluorescence channels. The result was expressed as mean fluorescence intensity.

Total RNA extraction and RT-PCR. Extraction and quality control of total cellular RNA were performed with standard procedures (3, 4). Four micrograms of total RNA were reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a 20-\(\mu\)l reaction mixture containing 50 ng random hexamer, 0.5 mM dNTPs, and 20 U RNase inhibitor. The cDNA was stored at \(-20^\circ\)C until further use. Gene expression of Fc-\(\alpha\) receptor 1 (Fc\(\alpha\)R or CD89), H1 and H2 chains of asialoglycoprotein receptor (ASGPR H1 and ASGPR H2), polymeric-immunoglobulin receptor (pIgR), Fc-\(\alpha\)/\(\mu\) chain receptor (Fc\(\mu\)R), transferrin receptor (TIR or CD71), TNF-\(\alpha\), and GAPDH by podocytes was examined by PCR using specific primers designed from known sequences described previously (3, 23). PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Images of the gel were captured using the Gel Doc 1000 gel documentation system from Bio-Rad (Hercules, CA). Gene expression of TNF-\(\alpha\) was then analyzed by the IP Lab gel software from Signal Analytics (Vienna, VA), and the results were expressed as a ratio to GAPDH amplicon. Full precaution was taken for all necessary steps to ensure the validity of the results as previously described (23).

Preparation of conditioned (spent) medium. HMC, podocytes, PTEC, and HUVEC were cultured with medium containing pIgA (final concentration of 50 \(\mu\)g/ml) isolated from IgAN patients or control subjects for 48 h. The concentration of IgA preparation used was selected based on our previous data that 50 \(\mu\)g/ml IgA was able to significantly increase the macrophage migration inhibitory factor by cultured HMC (19). The conditioned media (IgA-HMC conditioned medium, IgA-podocyte conditioned medium, IgA-PTEC conditioned medium, and IgA-HUVEC conditioned medium) were collected and stored at \(-70^\circ\)C until used. Conditioned medium from HMC cultured without the addition of IgA (control medium) was used as control.

Effect of IgA or IgA conditioned medium on the TNF-\(\alpha\) synthesis in podocytes. Growth-arrested HMC or podocytes were cultured in a sixwell culture plate (\(1 \times 10^6\) cells/well) with culture medium containing 0.5% FBS and incubated with IgA-HMC conditioned medium prepared from patients with IgAN or controls as described previously (19). Human mesangial cells or podocytes were grown to log phase and harvested by using 0.05% trypsin/0.02% EDTA for 5 min at room temperature. The cells were adjusted to \(5 \times 10^6\) per ml, and 200 \(\mu\)l of cell suspension were used in binding assays. Staining was performed at 4°C with staining buffer consisting of PBS with 1% FBS and 0.1% sodium azide. The cells were incubated with 100 \(\mu\)l of pIgA (final concentration of 50 \(\mu\)g/ml) for 30 min. After incubation, the cells were washed with staining buffer and then further incubated with 100 \(\mu\)l of rabbit anti-human IgA antibody. Background control staining was achieved by reaction with the preimmune F(ab\textsuperscript{2})\textsubscript{2} fragment of fluorescein-conjugated isotypic antibody. The stained cells were analyzed using a Coulter EPICS XL analyzer (Miami, FL). A minimum of 5,000 fixed cells for each sample was analyzed. Fluorescence intensity was evaluated by comparing the mean fluorescence channels. The result was expressed as mean fluorescence intensity.

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Specific primers for TNF-α receptor 1 and 2 (TNFR1 and TNFR2) and IL-6 were designed from known GeneBank sequences (TNFR1: NM_001065; TNFR2: PT019927; IL-6: NM_000600). The sequences of each primers were as follows: 1) TNFR1, sense primer 5’-TCA GTC CCG TGC CCA GTT CCA CCT T-3’ and antisense primer 5’-CTG AAG GGG GTT GGG GAT GGG GTC-3’, 2) TNFR2, sense primer 5’-GCT CCG GGC AAC ATG C-3’ and antisense primer 5’-GCC TTT CAC ACC AGC TCT GA-3’; and 3) IL-6, sense primer 5’-ATG AAC TTC TTC TCC ACA AGC GC-3’ and antisense primer 5’-GAA GAG CCC TCA GGC TGG ACT G-3’. The PCR products from TNF-α receptors or IL-6 and control (GAPDH) amplicons were mixed and separated by 1.5% wt/vol agarose gels, and the gel image was captured and analyzed as described in Total RNA extraction and RT-PCR. TNF-α receptor protein in cell lysates was determined by standard Western blotting as described previously (4).

To further determine the role played by the individual TNF-α receptor in the activation of podocytes, gene expression and protein synthesis of IL-6 were determined in podocytes incubated with IgA-HMC medium or TNF-α in the presence of different agonist and blocking antibodies against TNFR1 or TNFR2.

**Immunofluorescence examination.** Renal tissues were obtained from another 15 normotensive patients with mild IgAN (grade 1; Ref. 20) consecutively admitted for diagnostic renal biopsy with the presentation of microscopic hematuria. They had not previously received an angiotensin-converting enzyme inhibitor or ANG II receptor subtype-1 (ATR1) antagonist. Control renal tissues were obtained from the intact pole of kidneys removed for single circumcised tumor in eight normotensive subjects (comparable in age, sex, and race). The glomerular expression of nephrin plus TNFR1 or TNFR2 in renal biopsy specimens was detected by immunofluorescence staining using specific antibodies. Briefly, frozen renal sections were fixed with 2% paraformaldehyde for 10 min and were washed with PBS. Nonspecific binding was blocked by incubation of the slides for 30 min with Image-iT FX signal enhancer (Invitrogen, Eugene, OR). The sections were then incubated with guinea pig anti-nephrin (Fitzgerald Industries International, Concord, MA) plus mouse monoclonal anti-TNFRI or TNFR2 antibodies (10 μg/ml; containing 0.5% FBS and pIgA (final concentration of 50 μg/ml) isolated from patients with IgAN or control subjects for 48 h. The supernatants were collected and kept frozen at −70°C until assay for TNF-α. For examining the effect of IgA-HMC medium on podocytes, different preparations of IgA-HMC medium were diluted (8-fold for all experiments except 2- to 32-fold for dose-dependent experiments) with DMEM/F-12 containing 0.5% FBS for culturing podocytes. Growth-arrested podocytes were seeded onto a sixwell culture plate (1 × 10⁶ cells/well) and were cultured with diluted IgA-HMC medium for either 16 (for RT-PCR) or 48 h (for ELISA). After culture, the cells were collected for total RNA isolation and the supernatant was stored at −70°C before assay of TNF-α. To study whether TNF-α synthesis by podocytes was induced only via TNF-α receptors or IL-6 was no upregulation of TNF-α synthesis by podocytes after incubation with 25 pg/ml TNF-α day 4 and reached maximum on day 4. There was no upregulation of TNF-α synthesis by podocytes after incubation with 25 pg/ml TNF-α. *p < 0.05, when compared with data from day 0. Results are means ± SD from 6 individual experiments.

Effect of IgA-HMC medium on expression of TNF-α receptors by podocytes. Podocytes were cultured with pIgA preparation (50 μg/ml) or IgA-HMC medium for either 16 (for mRNA) or 48 h (for protein) unless otherwise stated. The cells were then collected for total RNA isolation for RT-PCR, and cell lysates were stored at −70°C before assay for TNF-α receptor protein.
tial, and the significance is defined as

buffer, pH 5.0 (19). Neutralizing antibodies to TNF-

were examined at high-power field. The preset grading criteria for glomerular TNF receptors staining were as follows: 0 if no podocyte staining, 1+ if <10% podocytes were positive; 2+ if 10 to <20% podocyte were positive; 3+ if 20 to <40% cells were positive; 4+ if 40 to <60% cells were positive; and 5+ if 60% or more cells were positive (20).

Reagents. Jacalin agarose was purchased from Pierce (Rockford, IL, USA). Rosewell Park Memorial Institute Medium (RPMI 1640 medium), DMEM/F-12 medium, and FBS were obtained from Life Technologies (Rockville, MD). The F(ab')2 fragment of FITC-conjugated rabbit anti-human IgA and FITC-conjugated preimmune rabbit Ig antibodies were obtained from Dako (Kyoto, Japan). Anti-secretory component was obtained from ICN Pharmaceuticals (Aurora, OH). Anti-CD89 blocking antibody (Clone My43) was obtained from Medarex (Annandale, NJ). Asialo-orosomucoid was prepared by desialylation of human orosomucoid with neuraminidase (0.03 U/mg protein) after incubation for 8 h at 37°C in 0.1 mol/l sodium acetate buffer, pH 5.0 (19). Neutralizing antibodies to TNF-α, IL-1β, IL-6, VEGF, TGF-β, HGF, PDGF, or basic FGF were obtained from R&D Systems (Minneapolis, MN). Blocking and agonist antibodies to TNF-receptor 1 or 2 (TNFR1 or TNFR2) were also obtained from R&D Systems. ELISA systems for measuring human TNF-α and IL-6 were obtained from Bender MedSystems (Vienna, Austria) and R&D Systems, respectively. All other chemicals were obtained from Sigma (St. Louis, MO).

Statistical analysis. All data were expressed as means ± SD unless otherwise specified. Statistical difference was analyzed with multivariate ANOVA for repeated measures. All P values quoted are two-tailed, and the significance is defined as P < 0.05.

RESULTS

Binding of IgA to PTEC and HMC. The binding (determined by flow cytometry) of pIgA from IgAN patients to HMC was higher than the binding of IgA from healthy controls (10.78 ± 2.78 vs. 7.29 ± 2.09; P < 0.001). However, with the use of the identical concentration of IgA and the same number of cells in the binding assay, the binding of IgA to podocytes were found to be only 7.2 and 8.9% of the binding of IgA to HMC for IgAN patients and healthy controls, respectively.

Expression of surface IgA receptors in podocytes. Table 1 summarizes the mRNA expression of FcεR1, H1 and H2 chains of ASGPR H1 and H2, plgR, Fcα/μR, and TRF in podocytes. Human mesangial cells and other cell lines were used as positive controls for gene expression of different receptors. Podocyte did not express mRNA for any known IgA receptors except the TRF. The IgA binding to podocyte was not blocked by preincubation with proteins that competitively blocked individual known IgA receptors including IgM, Anti-secretory component, asialo-orosomucoid, orosomucoid, anti-FcεR1 (clone My43), and transferrin (data not shown).

Synthesis of TNF-α by podocytes. The synthesis of TNF-α in podocytes did not differ between culture experiments with IgA preparations from IgAN patients, healthy controls, or disease controls (Fig. 1A). The synthesis of TNF-α was upregulated when mesangial cells were incubated with IgA preparations from IgAN patients when compared with IgA preparations from healthy controls (P < 0.001) or from disease controls (P < 0.01; Fig. 1B). We then explored whether conditioned medium from patients with IgAN exhibited any stimulatory effect on podocytes. The synthesis of TNF-α was upregulated when podocytes were incubated with IgA-HMC conditioned media from IgAN patients when compared with IgA-HMC conditioned media from healthy controls (P < 0.001). Similar findings were not observed...
with conditioned media prepared from podocytes, PTEC, or HUVEC incubating with pIgA from IgAN patients or healthy controls (Fig. 2).

Concentration of TNF-α, IL-1β, TGF-β, and PDGF in the IgA-HMC medium. The concentration of TNF-α in the IgA-HMC medium was determined to rule out the possibility that the level of TNF-α synthesis by podocytes after incubation with IgA-HMC medium was directly derived from the IgA-HMC medium. After an eightfold dilution, the calculated TNF-α concentration of the diluted spent medium used in culture experiments was 69.2 pg/ml. This concentration was less than one-seventeenth of the supernatant concentration of podocytes cultured with the diluted spent medium from HMC incubated with IgA from IgAN patients. The synthesis of TGF-β was upregulated in HMC incubated with IgA from patients with IgAN when compared with healthy controls (397.16 ± 49.60 vs. 308.89 ± 39.02 pg/ml; P = 0.0003). There was no induction of PDGF synthesis in HMC incubated with IgA from patients with IgAN or control subjects compared with basal PDGF level (data not shown). No IL-1β was detected in the medium of HMC incubated with IgA from patients or controls.

Effect of neutralizing antibodies in the synthesis of TNF-α by podocytes cultured with IgA-HMC medium. We then investigated the potential effect of a neutralizing anti-TNF-α antibody in modulating the synthesis of TNF-α incubated with diluted IgA-HMC medium from patients with IgAN. Anti-TNF-α neutralizing antibody at a concentration of 0.1 µg/ml almost completely abolished TNF-α synthesis induced by IgA-HMC medium from IgAN patients but not with neutralizing antibodies to IL-1β, IL-6, VEGF, TGF-β, HGF, PDGF, or FGF (Fig. 3).

Dose- and time-dependent effect of IgA-HMC medium on TNF-α synthesis by podocytes. The synthesis of TNF-α by podocytes cultured with IgA-HMC medium from patients with IgAN inversely correlated with the dilutions of IgA-HMC medium from patients with IgAN (Fig. 4). In parallel experiments, podocytes were cultured with an eightfold dilution of IgA-HMC medium for various time points (12 h to 12 days), the gene expression peaked at 24 h, while the protein synthesis was maximal around day 4 (Fig. 5). To test whether TNF-α in the IgA-HMC medium induces its synthesis by podocytes in an autocrine fashion, cultured podocytes were incubated with three dose concentrations of recombinant TNF-α (25, 50, or 100 pg/ml). Upregulation of TNF-α synthesis was only observed in experiments conducted with a concentration of 25 pg/ml and, similarly, the protein synthesis peaked around day 4 (Fig. 6).

Effect of IgA-HMC medium on expression of TNF-α receptors by podocytes. Both TNFR1 and TNFR2 were constitutively expressed in resting podocytes but only at a barely detectable level for the TNFR2 (Fig. 7). A higher expression of TNFR2 was inducible with IgA-HMC medium from patients with IgAN at low dilution or with TNF-α at 100 pg/ml. Incubation of podocytes with pIgA from patients

Fig. 8. Modulation of the expression of TNFR1 and TNFR2 mRNA and protein in podocytes by IgA-HMC conditioned medium. mRNA (A) and protein synthesis (B) of TNFR1 and TNFR2 were upregulated in podocytes cultured with IgA-HMC conditioned medium prepared from IgAN patients (solid bars) compared with that from healthy controls (open bars). There was no upregulation for either gene or protein expression of TNFR1 and TNFR2 in podocytes cultured directly with pIgA prepared from IgAN patients or healthy controls. The protein synthesis of TNFR1 and TNFR2 was upregulated by IgA-HMC (C) conditioned medium or TNF-α (D) in a dose-dependent manner. Results are means ± SD from 6 individual experiments.
with IgAN or healthy controls did not upregulate the expression of either receptor (Fig. 8). However, IgA-HMC medium from patients with IgAN upregulated the podocytic expression of either receptor in a dose-dependent manner but not with IgA-HMC medium from healthy controls. We then further examined the role played by individual TNF-α receptor in the activation of podocytes by determining the IL-6 synthesized by podocytes. IgA-HMC medium from patients with IgAN upregulated the IL6 synthesis by podocytes, and its stimulatory effect was abolished with either neutralizing antibody against TNF-α or blocking antibody against TNFR1 but not for TNFR2 (Figs. 9 and 10). IL-6 synthesis was enhanced in podocytes incubated with TNF-α or agonist antibody against TNFR1. The enhanced IL-6 synthesis induced by TNF-α was readily abolished by either anti-TNF-α neutralizing antibody or blocking antibody against TNFR1. In contrast, agonist antibody against TNFR2 exerted no enhancing effect and the blocking antibody against TNFR2 failed to abolish the TNF-α-induced IL-6 synthesis in podocytes. Similar findings were observed in TNF-α synthesis by podocytes.

**Glomerular expression of TNF receptors.** Glomerular immunoreactive TNFR1 was found on mesangial cells and podocytes from normal subjects and IgAN patients (Fig. 11A). Immunoreactive TNFR2 was expressed on mesangial cells and podocytes in glomeruli from IgAN patients but was barely detectable in glomeruli from normal subjects. The intensity of the slide preparations was scored independently by two pathologists without the knowledge of the nature of antibodies. In general, there was good concordance of the score and no sample had a discordance of score greater than one grade. Glomerular immunostaining for TNFR1 was significantly higher in patients with IgAN (mean score of 2.53 vs. 1.25 in control subjects; *P* = 0.0025; Fig. 11B). Similar findings were also detected in glomerular immunostaining for TNFR2 (mean score of 2.067 in IgAN vs. 0.25 in control subjects; *P* = 0.0003).

**DISCUSSION**

In IgAN, mesangial IgA deposition is an early event that subsequently leads to glomerular and tubular damages in a slow but progressive clinical course. The binding of IgA to HMC is increased in IgAN (10, 17). The interaction of IgA with glomerular mesangial cells induces cell proliferation (14) and the release of cytokines and chemokines (10, 12). Recently, we have shown that mediators (mainly TNF-α) released from HMC after mesangial IgA deposition can activate TEC leading to subsequent inflammatory changes in

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**Fig. 9.** IL-6 mRNA and TNF-α expression in podocytes cultured with IgA-HMC conditioned medium or recombinant TNF-α. A: IL-6 mRNA was upregulated when podocytes were cultured with IgA-HMC conditioned medium prepared from IgAN patients. The increased IL-6 mRNA expression can be partially blocked with anti-TNFFR1 blocking antibody or neutralizing anti-TNF-α but not with anti-TNFFR2 blocking antibody. Similar findings were observed with TNF-α mRNA (C). B: IL-6 mRNA was upregulated when podocytes were cultured with recombinant TNF-α or agonist anti-TNFFR1 antibody but not with anti-TNFFR2 agonist antibody. The increased IL-6 mRNA expression can be completely blocked with anti-TNFFR1 blocking antibody or neutralizing anti-TNF-α but not with anti-TNFFR2 blocking antibody. Similar findings were observed with TNF-α mRNA (D). Results are means ± SD from 6 individual experiments.
the tubulointerstitium (3). Our findings imply that humoral factors released from glomerular mesangium maintain a glomerulo-tubular cross talk in the development of tubulointerstitial damage in IgAN.

Podocytes are positioned strategically along the glomerulo-tubular axis, and there are scarce reports of podocyte abnormality in IgAN. However, their role in the pathogenesis of IgAN has not been studied. Necrosis and detachment of the podocytes from the glomerular basement membrane were observed in IgAN (27). The degree of podocytopenia was related with the severity of glomerular dysfunction (31). Complementing the histological findings of podocytopenia, patients with IgAN had increased urinary excretion of podocytes (21). Two available studies on the expression of nephrin in IgAN revealed conflicting data. Gagliardini et al. (11) detected a marked reduction of nephrin mRNA and extracellular nephrin in IgAN but not in minimal change nephropathy or focal segmental glomerulo-sclerosis. In contrast, Doublier et al. (7) found a reduction in nephrin and a shift of the podocyte-staining pattern only in IgAN patients with nephrotic syndrome but not in nonnephrotic IgAN patients.

In this study, we examined the pathophysiological effect of mediators released from mesangial cells triggered by IgA deposition on podocytes in the event of glomerulo-tubular cross talk using an in vitro system that we had previously used for studying the TEC function in IgAN. In cell culture experiments using an immortalized podocyte cell line, we observed binding of pIgA to podocytes but the quantity of IgA bound to podocytes was less than one-tenth that bound to HMC in IgAN. The lack of difference between binding capacities of IgA isolated from patients and healthy controls and the failure to upregulate the synthesis of TNF-α suggested such binding observed in the cell culture experiments was not specific. This is confirmed by the absence of any known IgA receptors except for the TIR in podocyte and the failure of blocking the IgA binding to podocyte with proteins that competitively blocked individual known IgA receptors including transferrin. Our present finding suggests TIR in podocytes behaves like that of TEC, which does not bind IgA or elicits any physiologic function (3). Aberrant glycosylation of O-glycans and the electrostatic interaction due to the anionic nature of the IgA molecule may be operative in the binding of IgA to HMC in IgAN (22, 24). Increased binding of IgA to various components of extracellular matrix has been demonstrated in IgAN (32). Whether similar mechanisms are operating for the nonspecific binding of IgA to cultured podocytes remains to be explored.

Based on our demonstration of the existence of a glomerulo-tubular cross talk in IgAN (3), we hypothesize that a similar glomerulo-podocytic cross talk existed in IgAN. Mesangial cells are found to communicate with podocytes through interferon-inducible protein-10 in Thy 1.1 glomerulonephritis (15). To confirm our hypothesis, we conducted podocyte culture experiments using conditioned medium from HMC preincubated with different IgA preparations. This medium transfer setting allowed no direct cell-cell communication but simulated the in vivo glomerulo-podocytic com-

Fig. 10. IL-6 (A, B) and TNF-α (C, D) protein synthesis in podocytes cultured with IgA-HMC conditioned medium or recombinant TNF-α. The findings in protein synthesis were similar to those of gene expression in Fig. 9.
Fig. 11. Immunofluorescence staining of TNFR1 and TNFR2 in kidney from normal subjects and IgAN patients. A: representative immunofluorescence staining of nephrin (red, first column) and TNFR1 or TNFR2 (green, second column) expression in glomeruli from normal subjects and IgAN patients (magnification X400). Glomerular immunoreactive TNFR1 was found on mesangial cells and podocytes from normal subjects and patients. Glomerular immunoreactive TNFR2 was expressed in glomeruli from IgAN patients but was barely detectable in glomeruli from normal subjects. The third column illustrates the results of image merging for nephrin and TNF receptor staining. Colocalization of nephrin and TNF receptor staining was indicated by arrowhead in the last column.

B: semiquantified glomerular immunostaining of TNF receptors using a five-point scale. Patients with IgAN are denoted with closed circles, and control subjects are denoted with open circles.
munication via humoral factors. Conditioned medium from HMC incubated with IgA from patients with IgAN, but not with IgA from disease or healthy controls, upregulated the synthesis of TNF-α. The increased synthesis of TNF-α after being cultured with the medium was not due to the “left over” from the HMC condition medium since the concentration of TNF-α in the supernatant of cultured podocytes was at least 17-fold higher than that in the diluted IgA-HMC medium. Activated mesangial cells produce cytokines and chemokines, including IL-1, IL-6, TNF-α, monocyte chemotactic protein-1, TGF-β, and PDGF (26). We speculate that these humoral factors/mediators from mesangial cells first activate the podocytes before reaching the tubulointerstitium either by glomerular filtration or by transportation via the postglomerular capillaries. Upon reaching the tubular compartment, these mediators could stimulate TEC to produce other proinflammatory cytokines and chemokines that eventually lead to tubular damage, interstitial mononuclear cell infiltration, and fibrosis (3, 12). Similar to our finding in the glomerulo-tubular cross talk in IgAN (3), TNF-α produced by HMC after stimulation by pIgA from IgAN patients leads to increased synthesis of TNF-α by podocytes in an autocrine fashion. Moreover, the result from culture experiments using different neutralizing antibodies suggests that TNF-α plays a unique and crucial role in mediating the inflammatory injury along the glomerulo-podocytic-tubular axis in IgAN.

The inability of differentiated podocytes to proliferate and repopulate the damaged glomerulus has been taken as the key factor in the progression of glomerular scarring (16). It is well believed that the disappearance of cell cycle promoters and a reciprocal upregulation of the cell cycle inhibitors, cyclin-dependent kinase inhibitors, coincide with the proliferation arrest and terminal differentiation of podocytes in the mature glomerulus. However, we have demonstrated that podocytes cultured with IgA-HMC medium or recombinant TNF-α can exhibit proinflammatory changes with increased synthesis of TNF-α and IL-6. These proinflammatory responses are both dose and time related. Our present findings together the recent observation that the renin-angiotensin system and VEGF are expressed in podocytes suggest these cells can play a proinflammatory role and may not be totally proliferation arrested (5, 25).

TNFR1 and TNFR2 are present in glomerular endothelium and TEC (1). Here, we have demonstrated that these receptors are also constitutively present in podocytes. Our histological study shows that both receptors are significantly upregulated in podocytes of IgAN patients. These receptors appear to play a distinct role in different disease entities. In an anti-glomerular basement membrane nephritis model, TNFR1 promotes systemic immune response and renal T-cell apoptosis while intrinsic cell TNFR2 regulates complement-dependent tissue injury (33). Despite the fact that these receptors are not inducible with pIgA isolated from patients with IgAN in vitro, their expression is readily upregulated by IgA-HMC medium from patients with IgAN or exogenous TNF-α. Our in vitro study suggests two functional roles played by the TNFR1 in podocytes after stimulation by IgA-HMC medium from patients with IgAN: IL-6 synthesis and apoptosis. IL-6 regulates the tubular ATR1 expression and enhances tubular ANG II production in IgAN (4). The interaction of ANG II and early expressed ATR1 will activate the protein kinase C and MAPK pathways, leading to inflammatory responses in TEC. Upregulation of TNFR1 in podocytes incubated with IgA-HMC medium may favor apoptotic cell death. Podocyte expression of proto-oncogene, Bcl-2, has previously been found to vary with the disease activity in IgAN (28). Downregulation of Bcl-2, associated with an increased ratio of Bax/Bcl-2 by glomerular epithelial cells, correlates with the severity of glomerulo-sclerosis. The upregulation of TNFR2 observed in our in vitro study suggests podocytes are in a chronic proinflammatory state in IgAN. On the basis of our recent data (3, 4) and the present study, we propose a hypothetical schema outlining the roles of podocytes in IgA-induced tubulointerstitial injury in IgAN (Fig. 12).

In conclusion, our finding suggests podocytes may play a contributory role in the development of interstitial damage in IgAN possibly by amplifying the TEC activation with enhanced TNF-α synthesis after inflammatory changes of HMC after IgA deposition. ATR1 and ATR2 denote ANG II receptor subtype-1 and subtype-2, respectively. *Mechanisms shown by previous studies; #mechanisms demonstrated in this study, and @denotes mechanisms speculated from these studies.

GRANTS

The study was supported by the Seed Funding for Basic Research of the University of Hong Kong (1159006) and the Research Grant Committee (Hong Kong; HKU 7678/07M). L. Chan was partially supported by L&T Charitable Foundation and the House of INDOCAFE.
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