ACTIVATION OF PODOCYTES BY MESANGIAL-DERIVED TNF-α: GLOMERULO-PODOCYTIC COMMUNICATION IN IgA NEPHROPATHY

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RUNNING HEAD: Podocyte activation in IgAN

ABBREVIATIONS: human mesangial cell: HMC; proximal tubular epithelial cell: PTEC; IgA nephropathy: IgAN; tumor necrosis factor-α: TNF-α

TEXT: 48 pages, 12 figures, 1 table, 33 references

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Abstract

We have previously documented that human mesangial cell (HMC)-derived tumor necrosis factor-α (TNF-α) is an important mediator involved in the glomerulotubular 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 hours significantly increased the gene expression and protein synthesis of TNF-α by podocytes with a seventeen-fold concentration above that of IgA-HMC medium. The up-regulation of TNF-α expression by podocyte was only abolished by a neutralizing antibody against TNF-α but not by other antibodies. Exogenous TNF-α up-regulated 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 receptor-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 following inflammatory changes of HMC.

KEY WORDS: IgA nephropathy, podocytes, tumor necrosis factor-α, mesangial cell, tubulointerstitial injury
Introduction

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 readily proliferate under normal conditions or in various renal diseases, likely the consequence of a robust expression or up-regulation 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. Normally, the glomerular barrier is impermeable to proteins.

IgA nephropathy (IgAN), the most common form of glomerulonephritis, 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) (17), proliferation of mesangial cells, increased synthesis of extracellular matrix (ECM) and infiltration of macrophage, 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 cells (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 discover a novel mechanism involving a glomerulo-tubular cross-talk (independent of proteinuria) in the development of tubulointerstitial damage in IgAN (3). Mediators [mainly tumor necrosis factor-α (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 human. All subjects (patients and healthy/disease controls) gave their written informed consent for blood or tissue collection. Twenty-two Chinese patients (10 male and 12 female) with clinical and renal immunopathological diagnosis of primary IgAN were studied. IgA nephropathy 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 more than 12 months and no significant renal impairment was documented. Systemic lupus erythematosus, Henoch-Schönlein purpura (HSP) 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 milliliter of blood was collected from each patient at clinical quiescence. The serum was isolated and frozen at –20°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, ten with minimal change nephropathy (MCNS) and five with HSP, were also recruited as disease controls. Serum was similarly collected from these individuals for processing.

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 (ATCC; Rockville, MD, USA). These cell lines, cultured under the recommended condition, were used as positive or negative control in various experiments. Human peripheral blood mononuclear cells (PBMC) 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 33°C, these cells grow into cobblestone morphology. Differentiated human podocytes that are grown at 37°C expressed markers of differentiated podocyte in vivo including nephrin, podocin, CD2AP, synaptopodin, P-cadherin and ZO-1. In all experiments, differentiated podocytes between passages 12 to 17 were used and were growth arrested with culture medium containing 0.5% FBS for 24 hours before the commencement of experiments.

**Isolation of polymeric IgA (pIgA) and flow cytometry**

Polymeric IgA1 (MW > 320kDa) 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 minutes at room temperature. The cells were adjusted to 5 x 10^6 per ml and 200 µl of cell suspension were used in binding assays. Staining was performed at 4°C with staining buffer consist of phosphate-buffered saline (PBS) with 1% FBS and 0.1% sodium azide.
The cells were incubated with 100 µl of pIgA (final concentration 50 µg/ml) for 30 minutes. After incubation, the cells were washed with staining buffer and then further incubated with 100 µl of rabbit anti-human IgA antibody. Background control staining was achieved by reaction with pre-immune F(ab′)2 fragment of fluorescein conjugated isotypic antibody. The stained cells were analysed using a Coulter EPICS XL analyser (Miami, Fl, USA). A minimum of 5,000 fixed cells for each sample was analysed. Fluorescence intensity was evaluated by comparing the mean fluorescence channels. The result was expressed as mean fluorescence intensity (MFI).

**Total RNA extraction and Reverse Transcription (RT)-Polymerase chain reaction (PCR)**

Extraction and quality control of total cellular RNA were performed with standard procedures (3,4). Four microgram of total RNA was reverse transcribed to cDNA with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) in a 20 µL reaction mixture containing 50 ng random hexamer, 0.5 mM dNTPs and 20 U of RNase inhibitor. The cDNA was stored at –20°C until further use. Gene expression of Fc alpha receptor 1 (FcαR or CD89), H1 and H2 chains of asialoglycoprotein receptor (ASGPR H1 and ASGPR H2), polymeric-immunoglobulin receptor (pIgR), Fc alpha/mu chain receptor (Fcα/µR), transferrin receptor (TfR or CD71), TNF-α, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) by podocytes were examined by PCR using specific primers designed from known sequences described previously (3,23). PCR products were analysed 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, USA). Gene expression of TNF-α was then
analysed by the IP Lab gel software from Signal Analytics (Vienna, VR, USA) 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**

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

**The effect of IgA or IgA conditioned medium on the TNF-α synthesis in podocytes**

Growth arrested HMC or podocytes were cultured in six-well culture plate (1 x 10^6 cells per well) with culture medium containing 0.5% FBS and pIgA (final concentration 50 µg/ml) isolated from patients with IgAN or control subjects for 48 hours. 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/F12 containing 0.5% FBS for culturing podocytes. Growth arrested podocytes were seeded onto six-well culture plate (1 x 10^6 cells per well) and were cultured with diluted IgA-HMC medium for either 16 (for RT-PCR) or 48 hours
(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. In order to study whether TNF-α synthesis by podocytes was induced only via TNF-α in the IgA-HMC spent medium, similar experiments were performed in cells incubated with antibodies against TNF-α (0.1 µg/ml), IL-1β (0.1 µg/ml), IL-6 (0.1 µg/ml), VEGF (0.1 µg/ml), TGF-β (10 µg/ml), HGF (0.1 µg/ml), PDGF (100 µg/ml), or FGF (0.1 µg/ml) 1 hour before stimulating with different spent medium.

Podocytes were cultured either with increasing dilution of IgA-HMC medium or with various time points (12 h to 12 days) in fixed concentration to determine the dose and time response in TNF-α synthesis. In parallel experiments, podocytes were cultured with increasing concentration of TNF-α for similar time period time points (12h to 12 days) to determine whether TNF-α were synthesized in an autocrine fashion.

**Effect of IgA-HMC medium on expression of TNF-α receptors by podocytes**

Podocytes were cultured with pIgA preparation (50µg/ml) or IgA-HMC medium for either 16 (for mRNA) or 48 hours (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.

Specific primers for TNF-R1, TNF-R2 and IL-6 were designed from known GeneBank sequences (TNF-R1 NM_001065; TNF-R2 PT019927; IL-6 NM_000600). The sequences of each primers were as follows: (i) TNF R1, sense primer 5’- TCA GTC CCG TGC CCA GTT CCA CCT T-3’ and anti-sense primer 5’- CTG AAG GGG GTT GGG GAT GGG GTC-3’, (ii) TNF R2, sense primer 5’- GCT CGC CGG GCC AAC ATG C-3’ and anti-sense primer 5’- GGC TTG CAC ACC ACG TCT GA-3’, and (iii)
IL-6, sense primer 5’- ATG AAC TCC TTC TCC ACA AGC GC-3’ and anti-sense 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 above. TNF-α receptor protein in cell lysates was determined by standard Western blotting as described previously (4).

To further determine the role played by 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 TNF-R1 or TNF-R2.

**Immunofluorescence examination**

Renal tissues were obtained from another fifteen normotensive patients with mild IgAN (grade 1) (20) consecutively admitted for diagnostic renal biopsy with the presentation of microscopic hematuria. They had not previously received angiotensin converting enzyme inhibitor or ATR1 antagonist. Control renal tissues were obtained from the intact pole of kidneys removed for single circumscribed tumor in eight normotensive subjects (comparable in age, sex, and race). The glomerular expression of nephrin plus TNF-R1 or TNF-R2 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. Non-specific binding was blocked by incubation of the slides for 30 min with Image-iT FX signal enhancer (Invitrogen, Eugene, OR, USA). The sections were then incubated with guinea pig anti-nephrin (Fitzgerald Industries International, Inc., Concord, MA, USA)
plus mouse monoclonal anti-TNF-R1 or TNF-R2 antibodies (R & D Systems; 10 μg/ml) overnight. The bound guinea pig anti-nephrin antibodies and mouse anti-TNF-R1 or -R2 antibodies were detect with Alexa Fluor 594 conjugated anti-guinea pig (Invitrogen) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Full precaution was taken for all necessary steps to ensure the specificity of the staining as previously described (20). There was complete absence of staining for all these negative control. Two renal pathologists without prior knowledge of clinical or laboratory data evaluated the expression of TNF receptors staining using an arbitrary 0–5+ scale. All glomeruli were examined at high-power field. The preset grading criteria for glomerular TNF receptors staining were as follows: zero 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), Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12 medium) and fetal bovine serum (FBS) were obtained from Life Technologies (Rockville, MD, USA). 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 (Anti-sc) was obtained from ICN Pharmaceuticals (Auror, OH, USA). Anti-CD89 blocking antibody (Clone My43) was obtained from Medarex (Annandale, NJ USA). Asialo-orosomucoid
(ASOR) was prepared by desialylation of human orosomucoid with neuraminidase (0.03 U/mg protein) following incubation for 8 hours at 37°C in 0.1 mol/L sodium acetate buffer, pH 5.0 (19). Neutralizing antibodies to tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), hepatic growth factor (HGF), platelet-derived growth factor (PDGF), or basic fibroblast growth factor (FGF) were obtained from R&D Systems (Minneapolis, MN, USA). Blocking and agonist antibodies to tumour necrosis factor-α receptor 1 or 2 (TNF-R1 or TNF-R2) 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, USA).

Statistical analysis

All data were expressed as means±standard deviation (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, using identical concentration of IgA and same number of cells in the binding assay, the binding of IgA to podocytes were 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 the FcαR, H1 and H2 chains of ASGPR, pIgR, Fcα/μR and the transferrin receptors (TfR) 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 transferrin receptor. The IgA binding to podocyte was not blocked by pre-incubation with proteins that competitively blocked individual known IgA receptors including IgM, anti-sc, ASOR, orosomucoid, anti-FcαR1 (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 [Figure 1A]. The synthesis of TNF-α was up-regulated 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) [Figure 1B]. We then explored whether conditioned medium from patients with IgAN exhibited any stimulatory effect on podocytes. The synthesis of TNF-α was up-regulated 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 [Figure 2].

**The 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. Following an eight-fold 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 up-regulated in HMC incubated with IgA from patients with IgAN when compared with healthy controls (397.16 ± 49.60 pg/ml 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 as 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 [Figure 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 [Figure 4]. In parallel experiments, podocytes were cultured with an eight-fold dilution of IgA-HMC medium for various time points (12h to 12 days), the gene expression peaked at 24 hours while the protein synthesis was maximal around day 4 [Figure 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). Up-regulation of TNF-α synthesis was only observed in experiments conducted with concentration > 25 pg/ml and, similarly, the protein synthesis peaked around day 4 [Figure 6].

**Effect of IgA-HMC medium on expression of TNF-α receptors by podocytes**

Both TNF-α receptors 1 and 2 were constitutively expressed in resting podocytes but only at a barely detectable level for the TNF-R2 [Figure 7]. A higher expression of TNF-R2 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 with IgAN or healthy controls did not up-regulate the expression of either receptor [Figure 8]. However, IgA-HMC medium from patients with IgAN up-regulated the podocytic expression of either receptor in a dose-dependent manner but not with IgA-HMC from health 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 up-regulated the IL6 synthesis by podocytes and its stimulatory effect was abolished with either neutralizing antibody against TNF-α or blocking antibody against TNF-R1 but not for TNF-R2 [Figures 9 and 10]. Interleukin 6 synthesis was enhanced in podocytes incubated with TNF-α or agonist antibody against TNF-R1. The enhanced IL-6 synthesis induced by TNF-α was readily abolished by either anti-TNF-α neutralizing antibody or blocking antibody against TNF-R1. In contrast, agonist antibody against TNF-R2 exerted no enhancing effect on and the blocking antibody against TNF-R2 failed to abolish the TNF-α-induced IL-6 synthesis in podocytes. Similar findings are observed in TNF-α synthesis by podocytes.

**Glomerular expression of TNF receptors**

Glomerular immunoreactive TNF-R1 was found on mesangial cells and podocytes from normal subjects and IgAN patients [Figure 11A]. Immunoreactive TNF-R2 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 TNF-R1 was significantly higher in patients with IgAN (mean score 2.53 versus 1.25 in control subjects, p = 0.0025) [Figure 11B]. Similar findings were also detected in glomerular immunostaining for TNF-R2 [mean score of 2.067 in IgAN versus 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 slowly 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 the tubulointerstitium (3). Our finding implicates 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 [GBM] was 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 and coworkers (11) detected a marked reduction of nephrin mRNA and extracellular nephrin in IgAN but not in minimal change nephropathy or focal segmental glomerulosclerosis. In contrast, Doublier and coworkers (7) found a reduction in nephrin and a shift of podocyte-staining pattern only in IgAN patients with nephrotic syndrome but not in non-nephrotic IgAN patients.
In this study, we examined the pathophysiological effect of mediator released from mesangial cells triggered by IgA deposition on podocytes in the event of glomerulo-tubular cross-talk using an \textit{in vitro} system that we had previously used for studying the TEC function in IgAN. In cell culture experiments using an immortalised 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 up-regulate the synthesis of TNF-\(\alpha\) 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 TfR in podocyte and failure of blocking the IgA binding to podocyte with proteins that competitively blocked individual known IgA receptors including transferrin. Our present finding suggests TfR 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 ECM had been demonstrated in IgAN (32). Whether similar mechanisms are operating for the non-specific binding of IgA to cultured podocytes remain 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 pre-incubated with
different IgA preparations. This medium transfer setting allowed no direct cell-cell communication but simulated the \textit{in vivo} glomerulo-podocytic communication via humoral factors. Conditioned medium from HMC incubated with IgA from patients with IgAN, but not with IgA from disease or healthy controls, up-regulated the synthesis of TNF-\(\alpha\). The increased synthesis of TNF-\(\alpha\) after culturing with the medium was not due to the ‘left over’ from the HMC condition medium since the concentration of TNF-\(\alpha\) 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-\(\alpha\), MCP-1, TGF-\(\beta\) 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 post-glomerular capillaries. Upon reaching the tubular compartment, these mediators could stimulate tubular epithelial cells to produce other pro-inflammatory cytokines and chemokines that eventually lead to tubular damage, interstitial mononuclear cells infiltration and fibrosis (3,12). Similar to our finding in the glomerulo-tubular cross-talk in IgAN (3), TNF-\(\alpha\) produced by HMC following stimulation by pIgA from IgAN patients leads to increased synthesis of TNF-\(\alpha\) by podocytes in an autocrine fashion. Moreover, the result from culture experiments using different neutralizing antibodies suggests that TNF-\(\alpha\) 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 up-regulation 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 pro-inflammatory changes with increased synthesis of TNF-α and IL-6. These pro-inflammatory responses are both dose- and time-related. Our present finding together the recent observation that the renin-angiotensin system and VEGF are expressed in podocytes suggest these cells can play a pro-inflammatory role and may not be totally proliferation arrested (5,25).

TNF-α receptor-1 (TNF-R1) and receptor-2 (TNF-R2) 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 up-regulated in podocytes of IgAN patients. These receptors appear to play a distinct role in different disease entities. In an anti-GBM nephritis model, TNF-R1 promotes systemic immune response and renal T cell apoptosis while intrinsic cell TNF-R2 regulates complement-dependent tissue injury (33). Despite these receptors are not inducible with pIgA isolated from patients with IgAN in vitro, their expression is readily up-regulated by IgA-HMC medium from patients with IgAN or exogenous TNF-α. Our in vitro study suggests two functional roles played by the TNF-R1 in podocytes following stimulation by IgA-HMC medium from patients with IgAN: IL-6 synthesis and apoptosis. Interleukin 6 regulates the tubular angiotensin II receptor subtype-1 (ATR1) expression and enhances tubular angiotensin II production in IgAN (4). The interaction of angiotensin II and early expressed ATR1 will activate the protein kinase C and MAPK pathways, leading to inflammatory responses in TEC. Up-regulation of TNF-R1 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). Down-regulation of Bcl-2, associated with an increased ratio of Bax/Bcl-2 by glomerular epithelial cells correlates with the severity of glomerulosclerosis. The up-regulation of TNF-R2 observed in our *in vitro* study suggests podocytes are in a chronic pro-inflammatory 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 [Figure 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 following inflammatory changes of HMC. *In vitro* study implicates humoral factors (predominantly TNF-α) released from glomerular mesangium are likely to maintain a glomerulo-podocytic cross-talk in the event of tubulointerstitial injury in IgAN.
ACKNOWLEDGMENT: Dr. Chan was partly supported by L & T Charitable Foundation and the House of INDOCAFE

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].

DISCLOSURES: None and no conflict of interest declared
References


Table 1. Semi-quantitative analysis of the mRNA expression of IgA receptors by RT-PCR (from zero to 3+)

<table>
<thead>
<tr>
<th>Amplicon size</th>
<th>FcαR</th>
<th>ASGPR H1</th>
<th>ASGPR H2</th>
<th>pIgR</th>
<th>TfR</th>
<th>Fcα/µR</th>
<th>GAPDH</th>
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Podocyte, HT29, HepG2, HMC, PBMC, U937, Negative control
Legends

Figure 1 Synthesis of TNF-α in HMC and podocyte cultured with IgA. (A) TNF-α synthesis was not increased in podocytes cultured with IgA prepared from patients with 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-α was up-regulated when mesangial cells were cultured with pIgA prepared from IgAN patients as when compared with healthy or disease controls. The concentration of TNF-α in the supernatant of mesangial cells cultured with pIgA prepared from healthy controls was 115.3±18.7 pg/ml.

Figure 2 Up-regulation of TNF-α mRNA and protein expression in podocytes cultured with IgA-HMC conditioned medium. The gene (A) and protein (B) expression of TNF-α were up-regulated in podocytes cultured with IgA-HMC conditioned medium prepared from IgAN patients (solid bar) when compared to that from healthy controls (open bar). Similar findings were not observed with podocytes cultured with conditioned medium prepared from podocytes, PTEC or HUVEC incubated with the same pIgA preparation. The results represent the mean±standard deviation from six individual experiments.

Figure 3 Inhibition assay. Effect of different neutralizing antibodies on inhibition of TNF-α gene (A) and protein (B) expression by podocytes following incubation with IgA-HMC conditioned medium prepared from patients with IgAN. TNF-α synthesis can only be blocked by neutralizing antibodies against TNF-α but not by antibodies to IL-1β, IL-6, VEGF, TGF-β, HGF,
PDGF or FGF. The results represent the mean ± standard deviation from six individual experiments.

**Figure 4** Dose-related synthesis of TNF-α. (A) TNF-α gene expression and (B) protein synthesis increased significantly in podocytes following incubation with different doses (2- to 16-fold diluted medium) of IgA-HMC conditioned medium prepared from IgAN patients (solid bar). There was no change with IgA-HMC conditioned medium prepared from control (open bar) * signifies p < 0.05 when compared with data from day 0. The results represent the mean ± standard deviation from six individual experiments.

**Figure 5** Time-related synthesis of TNF-α. (A) TNF-α gene expression increased significantly in podocytes following incubation with IgA-HMC conditioned medium prepared from IgAN patients (closed circles) and reached maximum on day 1 and then fell close to basal level from day 4 onward. There was no change in TNF-α gene expression by podocytes following incubation with IgA-HMC conditioned medium prepared from healthy controls (opened circles) (B) The synthesis of TNF-α was significantly up-regulated in podocytes following incubation with IgA-HMC conditioned medium prepared from IgAN patients (closed circles) from day 1 and reached maximum on day 4. There was such changes with IgA-HMC conditioned medium prepared from healthy controls (opened circles) * signifies p < 0.05 when compared with data from day 0. The results represent the mean ± standard deviation from six individual experiments.

**Figure 6** Auto-amplification of TNF-α synthesis by podocytes. The synthesis of
TNF-α was significantly up-regulated in podocytes following incubation with TNF-α (50 and 100 pg/ml) from day 1 and reached maximum on day 4. There was no up-regulation of TNF-α synthesis by podocyte following incubation with 25 pg/ml TNF-α. * signifies p < 0.05 when compared with data from day 0. The results represent the mean + standard deviation from six individual experiments.

**Figure 7** Expression of TNF-R1 and TNF-R2 mRNA and protein in podocytes. (A) Gene expression of TNF-R1 and TNF-R2 mRNA determined by RT-PCR. There was constitutive expression of TNF-R1 with barely detectable TNF-R2. TNF-α and IgA-HMC conditioned medium up-regulated TNF-R1 and TNF-R2 mRNA expression. (B) Similar findings were observed for TNF-R1 and TNF-R2 protein determined by immunoblotting. The results represent the mean ± standard deviation from six individual experiments.

**Figure 8** Modulation of the expression of TNF-R1 and TNF-R2 mRNA and protein in podocytes by IgA-HMC conditioned medium. The (A) mRNA and (B) protein synthesis of TNF-R1 and TNF-R2 were up-regulated in podocytes cultured with IgA-HMC conditioned medium prepared from IgAN patients (solid bar) when compared to that from healthy controls (open bar). There was no up-regulation for either gene or protein expression of TNF-R1 and TNF-R2 in podocytes cultured directly with pIgA prepared from IgAN patients or healthy controls. The protein synthesis of TNF-R1 and TNF-R2 was up-regulated by (C) IgA-HMC conditioned medium or (D) TNF-α in a dose-dependent manner. The results represent the mean ± standard deviation from
six individual experiments.

**Figure 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 up-regulated 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-TNF-R1 blocking antibody or neutralizing anti-TNF-α but not with anti-TNF-R2 blocking antibody. Similar findings were observed with TNF-α mRNA (C). (B) IL-6 mRNA was up-regulated when podocytes were cultured with recombinant TNF-α or agonist anti-TNF-R1 antibody but not with anti-TNF-R2 agonist antibody. The increased IL-6 mRNA expression can be completely blocked with anti-TNF-R1 blocking antibody or neutralizing anti-TNF-α but not with anti-TNF-R2 blocking antibody. Similar findings were observed with TNF-α mRNA (D). The results represent the mean±standard deviation from six individual experiments.

**Figure 10** IL-6 and TNF-α 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 figure 9.

**Figure 11** Immunofluorescence staining of TNF-R1 and TNF-R2 in kidney from normal subjects and IgAN patients. (A) Representative immunofluorescence staining of nephrin (red, first column) and TNF-R1 or TNF-R2 (green, second column) expression in glomeruli from normal subjects and IgAN patients [magnification x 400]. Glomerular immunoreactive TNF-R1 was found on mesangial cells and podocytes from normal subjects and
patients. Glomerular immunoreactive TNF-R2 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 receptors staining. Co-localization of nephrin and TNF receptors staining was indicated by arrow head in the last column. (B) Semi-quantified 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.

Figure 12. A schema of various mechanisms operating between the HMC, podocytes, and TEC following mesangial IgA deposition in the development of tubulointerstitial injury in progressive IgAN. TNF-α released from the glomerular mesangium following IgA deposition induced TNF-α synthesis by the podocytes. Podocyte-derived TNF-α further up-regulated the TNF-α production in an autocrine manner. TNF-α derived from mesangial cells and podocytes up-regulated the expression of TNF receptors. The binding to TNF-R1 leads to IL-6 synthesis and apoptosis while binding to TNF-R2 maintains pro-inflammatory cellular responses. Podocytes played a contributory role in the development of interstitial damage in IgAN by amplifying the TEC activation with enhanced TNF-α synthesis following inflammatory changes of HMC after IgA deposition. ATR1 and ATR2 denote angiotensin II receptor subtype-1 and subtype-2 respectively. * denotes mechanisms shown by previous studies, # denotes mechanisms demonstrated in this study, and @ denotes mechanisms speculated from these studies.
Figure 1
Figure 2
Figure 3
Figure 4

A

B
Figure 5
Figure 6

(A) 

(B)
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**A**

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**B**

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Figure 7
Figure 8
Figure 8

C

D

* p < 0.01 v.s. medium control
Figure 9

A

B

* p < 0.01 v.s. medium control  Ω p < 0.01 v.s. IgA-HMC conditioned medium  # p < 0.01 v.s. TNF-α treatment
Figure 9
Figure 10

A

TNF R1 blocking antibody
TNF R2 blocking antibody
Neutralizing TNF-α antibody
IgA-HMC conditioned medium

B

TNF R1 agonist antibody
TNF R1 blocking antibody
TNF R2 agonist antibody
TNF R2 blocking antibody
Neutralizing TNF-α antibody
TNF-α

* p < 0.01 v.s. medium control  Ω p < 0.01 v.s. IgA-HMC conditioned medium  # p < 0.01 v.s. TNF-α treatment
C

![Bar graph showing TNF-α levels with different antibody treatments.](image)

- TNF R1 blocking antibody
- TNF R2 blocking antibody
- Neutralizing TNF-α antibody
- IgA-HMC conditioned medium

D

![Bar graph showing TNF-α levels with different antibody treatments.](image)

- TNF R1 agonist antibody
- TNF R1 blocking antibody
- TNF R2 agonist antibody
- TNF R2 blocking antibody
- Neutralizing TNF-α antibody
- TNF-α

* p < 0.01 v.s. medium control  Ω p < 0.01 v.s. IgA-HMC conditioned medium  # p < 0.01 v.s. TNF-α treatment

Figure 10
A

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B

![Graph showing comparison between TNF-R1 and TNF-R2](image7)

**Figure 11**
humoral factors (TNF-α)

glomerulo-tubular cross-talk via TNF-α, IL-6 and angiotensin II

tubulo-glomerular cross-talk favoring glomerulosclerosis

proteinuria

pro-inflammatory cellular response

Apoptosis

TNF-α

IL-6

IgA

no IgA binding

Figure 12