Podocyte injury induced by mesangial-derived cytokines in IgA nephropathy

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Abstract

Background. We have previously documented that human mesangial cell (HMC)-derived tumour necrosis factor-α (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 function of podocytes in IgAN.

Methods. Podocyte markers were examined in renal tissues by immunofluorescence. In vitro experiments were conducted with podocytes cultured with polymeric IgA (pIgA) or conditioned medium prepared from HMC incubated with pIgA (IgA–HMC conditioned medium).

Results. Glomerular immunostaining for nephrin or ezrin was significantly weaker in patients with IgAN. The immunostaining of IgA and nephrin was distinctly separate with no co-localization. In vitro experiments revealed no effect of pIgA on the expression of these podocyte proteins as IgA from IgAN patients did not bind to podocytes. In contrast, IgA conditioned medium prepared from IgAN patients down-regulated the expression of these podocyte proteins as well as other podocyte markers (podocin and synaptopodin) in cultured podocytes. The mRNA expression of nephrin, ezrin, podocin but not synaptopodin correlated with the degree of proteinuria and creatinine clearance. The down-regulation was reproducible in podocytes cultured with TNF-α or transforming growth factor-β (TGF-β) at concentration comparable to that in the IgA–HMC conditioned medium. The expression of these podocyte proteins was restored partially with a neutralizing antibody against TNF-α or TGF-β and fully with combination of both antibodies.

Conclusion. Our finding suggests podocyte markers are reduced in IgAN. An in vitro study implicates that humoral factors (predominantly TNF-α and TGF-β) released from mesangial cells are likely to alter the glomerular permeability in the event of proteinuria and tubulointerstitial injury in IgAN.

Keywords: IgA nephropathy; podocytes; slit diaphragm; transforming growth factor-β; tumour necrosis factor-α

Introduction

IgA nephropathy (IgAN) remains 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), proliferation of mesangial cells, increased synthesis of extracellular matrix (ECM) and infiltration of macrophage, monocytes and T cells. The severity of tubulointerstitial damage in IgAN correlates closely with the declining renal function and the long-term clinical outcome [1]. However, IgA is rarely deposited in the tubulointerstitium in IgAN and this is related to the absence of known IgA receptors in the tubular epithelial cells (TEC) [2]. Recently, we discovered a novel mechanism involving a glomerulo-tubular cross-talk (independent of proteinuria) in the development of tubulointerstitial damage in IgAN [2]. Mediators [mainly tumour 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 podocyte pathology in IgAN. The pathophysiological effects on podocytes by mediators released from mesangial cells triggered by IgA deposition were further investigated.

Materials and methods

Reagents

Jacalin agarose was purchased from Pierce (Rockford, IL, USA), Rosewell Park Memorial Institute Medium (RPMI...
bovine serum (FBS) were obtained from Life Technologies (Rockville, MD, USA). F(ab)2 fragment of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgA was obtained from Dako (Kyo, Tokyo, Japan). Neutralizing antibodies to TNF-α, interleukin-1β (IL-1β), 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). Antibody to nephrin was obtained from Fitzgerald Industries International (Concord, MA, USA). Antibody to ezrin was obtained from Epitomics Inc. (Burlingame, CA, USA). Antibodies to secreted protein acidic and rich in cysteine (SPARC) and synaptopodin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horse radish peroxidase (HRP)-conjugated rabbit anti-guinea pig IgG was obtained from Abcam (Cambridge, UK) and other secondary antibodies for immunoblotting were obtained from Dako. All other chemicals (including antibody to podocin) were obtained from Sigma (St Louis, MO, USA).

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 males and 12 females) 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 and occasionally along the peripheral capillary basement membrane 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-Schonlein purpura (HSP) and hepatic diseases were excluded by detailed clinical history, examination and negative laboratory findings for hypocomplementaemia, anti-DNA antibody or hepatitis B virus surface antigen. Twenty millilitres of blood was collected from each patient at clinical quiescence. The serum was isolated and frozen at −20°C until isolation of IgA. Daily urinary protein and creatinine clearance were determined at the same time of serum collection. Every IgA preparation was separately obtained from each individual patient or healthy control.

Fifteen healthy subjects (7 males and 8 females), comparable in age and race, with no microscopic haematuria or proteinuria, were recruited as normal controls. Serum was similarly collected from these individuals for processing.

Immunofluorescence examination
Renal tissues were obtained from another 15 normotensive patients with mild IgAN (grade 1) [3] consecutively admitted for diagnostic renal biopsy with the presentation of microscopic haematuria. 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 tumour in eight normotensive subjects (comparable in age, sex and race). The glomerular localization of IgA deposits and podocyte markers (nephrin, ezrin or SPARC) 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 the Image-iT FX signal enhancer (Invitrogen, Eugene, OR, USA). The sections were then incubated with guinea pig anti-nephrin (1:30), rabbit anti-ezrin (1:200) or mouse anti-SPARC (1:50) overnight. The bound guinea pig anti-nephrin antibody, rabbit anti-ezrin antibody and mouse anti-SPARC antibody were detected with Alexa Fluor 594 conjugated secondary antibodies (Invitrogen). In some experiments, sections stained with anti-nephrin were further incubated with FITC-conjugated anti-human IgA antibodies to determine whether IgA was deposited in podocytes. Full precaution was taken for all necessary steps to ensure the specificity of the staining as previously described [4]. There was a complete absence of staining for all these negative controls. Two renal pathologists without prior knowledge of clinical or laboratory data evaluated the expression of nephrin or ezrin staining using an arbitrary 0–5+ scale. All glomeruli were examined at high-power field. The preset grading criteria for glomerular nephrin or ezrin staining were as follows: 0 if no podocyte staining, 1+ if <10% podocytes were positive; 2+ if 10% to <20% podocytes 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 [2].

Cell culture and isolation of pIgA
Isolation, characterization and culture of human mesangial cells (HMC), proximal TEC (PTEC) and human umbilical vein endothelial cells (HUVEC) were performed as previously described [4]. A conditionally immortalized human podocyte cell line was established in our laboratory by transfection with a temperature-sensitive SV40-T gene [5]. 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 and 17 were used and were growth arrested with culture medium containing 0.5% FBS for 24 h before the commencement of experiments.

pIgA1 (MW > 320 kDa) was isolated and purified from sera of patients with IgAN or controls as described previously [2]. Analysis of the glycosylation profile revealed that the anionic fraction of these pIgA1 preparations was underglycosylated with higher content of α(2,6)-linked sialic acid [6].
Preparation of conditioned (spent) medium
Conditioned (spent) medium was prepared by incubating HMC, podocytes, PTEC and HUVEC with the medium containing pIgA (final concentration 50 µg/ml) isolated from IgAN patients or control subjects for 48 h. The use of conditioned medium was based on our recent findings that pIgA from IgAN patients does not activate podocytes directly, and the absence of known IgA receptor in podocytes that binds IgA [7]. 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 [3]. 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°C until used. ‘Medium control’ is defined as the conditioned medium from HMC cultured without the addition of IgA and was used as control in all in vitro experiments.

Viability and apoptosis of podocytes cultured with the IgA–HMC conditioned medium
The cytotoxic effect of the IgA–HMC conditioned medium on podocyte was determined by the release of lactate dehydrogenase (LDH) (LDH assay kit, Roche Diagnostics, Indianapolis, IN, USA). Results were expressed as percentage changes in relative LDH release (absorbance ratio between the LDH release and the total intracellular LDH) compared with that of the medium control. To examine
Fig. 2. Down-regulation of nephrin and ezrin mRNA and protein expression in podocytes cultured with the IgA–HMC conditioned medium. The gene (A, C) and protein (B, D) expressions of nephrin and ezrin were down-regulated in podocytes cultured with the IgA–HMC conditioned medium prepared from IgAN patients (black bar) when compared to that from healthy controls (grey bar). Similar findings were not observed in podocytes cultured with the 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.

whether the IgA–HMC conditioned medium induced apoptosis in podocyte, activation of caspase 3 was determined using a caspase 3 activity fluorometric immunosorbant enzyme assay (Roche Diagnostics).

The effect of the IgA or IgA conditioned medium on the expression of podocyte markers

Growth-arrested podocytes were cultured in a six-well culture plate (1 × 10⁶ 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 either 4 (for RT–PCR) or 48 h (for western blotting). After culture, cells were collected for total RNA isolation or cell lysate preparation.

To examine the effect of the IgA–HMC conditioned medium on podocytes, different preparations of the IgA–HMC conditioned medium were diluted (8-fold for all experiments except 4- to 128-fold for dose-dependent experiments) with DMEM/F12 containing 0.5% FBS for use as culture medium. Growth-arrested podocytes were seeded onto a six-well culture plate (1 × 10⁶ cells per well) and were cultured with the diluted IgA–HMC conditioned medium for either 4 (for RT–PCR) or 48 h (for western blotting). After culture, cells were collected for total RNA isolation or cell lysate preparation.

Quantification of nephrin, ezrin, GLEPP1 and SPARC mRNA expression by real-time PCR

Gene expression of these podocyte markers was determined using real-time quantitative polymerase chain reaction. GAPDH was used as the normalizing gene. Nephrin qPCR primers used were: forward—5′CAACTGGGAGAGAATGGGAGAA3′; reverse—5′AATCTGACAACAAGACGAGCA3′; ezrin qPCR primers used were: forward—5′ACGTCTGAGAAATCAACAGC3′; reverse—5′TTCTCCTC
Fig. 3. Down-regulation of podocin and synaptopodin mRNA and protein expression in podocytes cultured with the IgA–HMC conditioned medium. The gene (A, C) and protein (B, D) expression of podocin and synaptopodin were down-regulated in podocytes cultured with the IgA–HMC conditioned medium prepared from IgAN patients (black bar) when compared to that from healthy controls (grey bar). The results represent the mean ± standard deviation from six individual experiments.

ATAGTC-CTGCAG3′; SPARC qPCR primers used were: forward—5′CAAGAAAGCCCTGCC-TGATGAG A3′; reverse—5′GGGGGTGTTTCTCTCATCCAGC3′; podocin qPCR primers used were: forward—5′AAGAGTA ATTATTTCCGACTGGGACAT3′; reverse—5′TGTCATCGATCTCAAAAGG3′; synaptopodin qPCR primers used were: forward—5′CCCAAGGTGACCCGAAT3′; reverse—5′CTGCCGCCGCTTCTCA3′; GAPDH qPCR primers used were: forward—5′TGCCACTCAGAAGACTGTG G3′; reverse—5′GGATGCGAGGG-ATGATGTTC3′. Following DNase treatment of total RNA (2 µg), cDNA was synthesized in 20 µl reactions using the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) and stored at −20°C until use. Real-time PCR amplification was performed in the ABI Prism 7500 Sequence Detection system using the SYBR-Green reaction kit (Applied Biosystems, Foster City, CA, USA). The data obtained were analysed using the comparative C_T (cycle threshold) method. To determine the quantity of the podocyte marker transcripts present in podocytes from various groups, the C_T values were first normalized by subtracting the C_T value obtained from the GAPDH control (ΔC_T = C_T, nephrin, ezrin, podocin, synaptopodin or SPARC − C_T, GAPDH). Relative mRNA fold changes of these podocyte markers were calculated by subtracting the normalized C_T values obtained for various experimental groups (exp) relative to the medium control (ctl) [(exp − ΔΔC_T, ctl)] and the relative podocyte markers mRNA fold changes was determined (2^−ΔΔC_T).

Western blot analysis
Podocytes were lysed with a lysis buffer containing protease inhibitor cocktails (Sigma). The cell extracts were pelleted at 15 000 g for 10 min to remove cell debris. The protein concentrations were measured by a modified Lowry method using bovine serum albumin as standard (DC protein assay kit, BioRad). Ten micrograms of total protein from 10^6 cells was electrophoresed through a 15% SDS–PAGE gel before transferring to a PVDF membrane. After blocking for 1 h at room temperature in a blocking buffer (1% gelatin in PBS with 0.05% Tween-20), the membrane was incubated for 16 h with a monoclonal anti-actin antibody (1:1000), guinea pig anti-nephrin (1:200), rabbit anti-ezrin (1:5000), anti-podocin (1:200), anti-synaptopodin (1:200) or anti-SPARC (1:200) in PBS–Twee. The membrane was washed and incubated for 2 h at room temperature with a peroxidase-labelled rabbit anti-guinea pig, goat anti-rabbit or goat anti-mouse immunoglobulin (Dako). After further washing, the membrane was detected with ECL chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). For semi-quantitative determination of protein expression, western blotting images for some experiments were scanned on a flatbed scanner and the density of
Fig. 4. Assay for cytotoxicity and apoptosis. (A) Results of LDH release from podocyte cultured with the IgA–HMC conditioned medium. No cytotoxicity was demonstrated in podocytes exposed to various dilutions of the IgA–HMC conditioned medium prepared from control or the 4- and 8-fold diluted IgA–HMC conditioned medium prepared from IgAN patients. There was increased LDH release when podocyte was cultured with the twofold diluted IgA–HMC conditioned medium prepared from IgAN patients. Results were expressed as percentage changes in relative LDH release (absorbance ratio between the LDH release and the total intracellular LDH) compared with that of the medium control. Podocyte incubated with 100 µM of tamoxifen was used as a positive control. 

(B) Results of caspase 3 assay indicate no apoptosis in podocytes exposed to various dilutions of the IgA–HMC conditioned medium prepared from control or 4- and 8-fold diluted IgA–HMC conditioned medium prepared from IgAN patients. There was an increase in caspase 3 activity when podocyte was cultured with the twofold diluted IgA–HMC conditioned medium prepared from IgAN patients. UV-irradiated podocytes served as a positive control. All results represent mean ± standard deviation of 10 patients and controls.

the bands was quantitated using the ImageQuant software (Molecular Dynamic, Sunnyvale, CA, USA). Densitometry results were reported as percentages of medium control after normalization with the average arbitrary integrated values of the actin signal.

Statistical analysis

All data were expressed as means ± standard deviation unless otherwise specified. Statistical difference was analysed with multivariate ANOVA for repeated measures. Correlation analysis was done by Spearman’s method. All P-values quoted are two-tailed and the significance is defined as P < 0.05.

Results

Glomerular expression of podocyte markers

IgA was deposited in glomerular mesangium in renal tissue from patients with IgAN [Figure 1A(a)] but from normal subjects. Glomerular immunoreactive nephrin was found on podocytes in renal tissues from both normal subjects and IgAN patients [Figure 1A(c and d)]. The immunostaining of IgA and nephrin was distinctly separate with no co-localization [Figure 1A(b)]. Glomerular immunostaining for nephrin was weak in patients with IgAN [Figure 1A(d)] with a significantly lower mean score ($P = 0.001$) (Figure 1B). Similarly, glomerular immunostaining for ezrin was weaker in patients with IgAN [Figure 1A(f)] with a lower mean score ($P = 0.002$) (Figure 1B). Immunoreactive SPARC was barely detectable...
in the glomerulus (data not shown). There was no difference in mean score for SPARC between renal tissues from normal subjects and IgAN patients. 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.

Expression of podocyte markers in podocyte incubated with pIgA preparation or different conditioned media

Following the histological finding of reduced podocytic expression of nephrin and ezrin in IgAN, we next conducted an in vitro study to determine whether pIgA isolated from IgAN specifically down-regulated the expression of these podocyte proteins and other podocyte markers (including podocin and synaptopodin) in cultured podocytes. No change in the expression of podocyte markers was observed following incubating podocytes with different pIgA preparations (data not shown). We then explored whether the conditioned medium from patients with IgAN exhibited any down-regulatory effect on podocytes, as mediators released from mesangial cells after IgA deposition activate TEC and lead to subsequent inflammatory changes in the renal interstitium in IgAN [2]. The gene and protein expressions of nephrin and ezrin were down-regulated when podocytes incubated with the IgA–HMC conditioned medium from IgAN patients were compared with those incubated with the IgA–HMC conditioned medium from healthy controls ($P < 0.001$). Similar findings were not observed with conditioned media prepared from podocytes, PTEC or HUVEC incubated with pIgA from IgAN patients or healthy controls (Figure 2). The IgA–HMC conditioned medium from IgAN patients also down-regulated other podocyte proteins including podocin and synaptopodin (Figure 3). However, the IgA–HMC conditioned medium from IgAN patients did not exert any modulatory effect on SPARC in cultured podocytes (data not shown). There was no increase in LDH or caspase 3 release in podocytes exposed to the IgA–HMC conditioned medium with greater than twofold dilution suggesting that the down-regulation of these podocyte markers was not due to the cytotoxic or apoptotic effect of the IgA–HMC conditioned medium (Figure 4). The mRNA expression of nephrin, ezrin, podocin but not synaptopodin correlated with the degree of proteinuria and creatinine clearance (Figure 5).

Dose- and time-dependent effect of the IgA–HMC conditioned medium on nephrin and ezrin expressions in podocytes

The down-regulation of nephrin or ezrin expression by podocytes cultured with the IgA–HMC conditioned medium from patients with IgAN correlated inversely with the dilutions of the IgA–HMC conditioned medium.
Podocyte injury in IgAN

Fig. 8. Effect of different neutralizing antibodies on the expression of nephrin and ezrin. Gene (A, C) and protein (B, D) expressions of nephrin or ezrin by podocytes following incubation with the IgA–HMC conditioned medium prepared from patients with IgAN. Expression of nephrin and ezrin can only be partially restored by neutralizing antibodies against TNF-α or TGF-β, but not by antibodies to IL-1β, IL-6, VEGF, HGF, PDGF or FGF. * and ** signify $P < 0.01$ or $P < 0.05$ respectively when compared with data from podocytes cultured with the IgA–HMC conditioned medium. The results represent the mean ± standard deviation from six individual experiments. The IgA–HMC medium abbreviates for the IgA–HMC conditioned medium.

Fig. 9. Inhibition assay. Effect of neutralizing antibodies against TNF-α or TGF-β gene (A, C) and protein (B, D) expressions for nephrin or ezrin by podocytes following incubation with the IgA–HMC conditioned medium prepared from patients with IgAN, recombinant TNF-α or recombinant TGF-β. Down-regulation of nephrin or ezrin expression by the IgA–HMC conditioned medium can only be partially ameliorated by the presence of neutralizing antibodies against TNF-α or TGF-β. The expression of nephrin or ezrin was completely restored with combination of both neutralizing antibodies. Recombinant TNF-α or TGF-β down-regulated the expression of nephrin or ezrin and this was restored by the presence of corresponding neutralizing antibody. * and ** signify $P < 0.01$ and $P < 0.05$ respectively when compared with data from podocytes cultured with similar medium in the absence of neutralizing antibodies. The results represent the mean ± standard deviation from six individual experiments. The IgA–HMC medium abbreviates for the IgA–HMC conditioned medium.
from patients with IgAN at 16-fold dilution and below (Figure 6). In parallel experiments, podocytes were cultured with an eightfold dilution of the IgA–HMC conditioned medium for various time points (2–72 h). The gene and protein expressions were significantly reduced at 4 and 24 h respectively with a further stepwise time-dependent reduction (Figure 7).

**Effect of neutralizing antibodies in the down-regulation of nephrin or ezrin in podocytes cultured with the IgA–HMC conditioned medium**

We then investigated the potential counteracting effect of different neutralizing antibodies against mediators that may be released from activated mesangial cells. The anti-TNF-α neutralizing antibody at a concentration of 0.1 µg/ml and the anti-TGF-β neutralizing antibody at a concentration of 10 µg/ml partially abolished the down-regulatory effect of nephrin or ezrin expression induced by the IgA–HMC conditioned medium from IgAN patients, but not with neutralizing antibodies to IL-1β, IL-6, VEGF, HGF, PDGF or FGF (Figure 8). Combination of anti-TNF-α and anti-TGF-β neutralizing antibodies completely abolished the down-regulatory effect induced by the IgA–HMC conditioned medium from IgAN patients. The concentration of TNF-α and TGF-β in the IgA–HMC medium was determined by standard ELISA [3,5]. Following an eightfold dilution, the calculated TNF-α and TGF-β concentration of the diluted conditioned medium used in culture experiments was 69.2 pg/ml and 2.8 ng/ml, respectively.

**Effect of recombinant TNF-α or TGF-β on nephrin or ezrin expressions in podocytes**

Finally, following the data from neutralizing antibodies, we tested whether TNF-α and/or TGF-β are predominant mediators in the IgA–HMC conditioned medium responsible for the down-regulation of nephrin and ezrin in cultured podocytes. Recombinant TNF-α and TGF-β, both at concentration comparable to that in eightfold dilution of the IgA–HMC conditioned medium, reduced the expression of these markers by ∼40%, similar to that of the diluted IgA–HMC conditioned medium (Figure 9). Down-regulation of nephrin and ezrin expression induced by TNF-α or TGF-β was completely abolished by the neutralizing anti-TNF-α or anti-TGF-β antibody, respectively. However, anti-TNF-α exerted no effect on the down-regulation of these podocyte markers induced by TGF-β and vice versa for anti-TGF-β and TNF-α.

**Discussion**

Podocytes are positioned strategically along the glomerulotubular axis. There are scarce reports of podocyte abnormality in IgAN and their role in the pathogenesis of IgAN has rarely been studied. Necrosis and detachment of the podocytes from the glomerular basement membrane was observed in IgAN [8]. The degree of podocytopaenia was related to the severity of glomerular dysfunction [9]. Complementing the histological findings of podocytopaenia, patients with IgAN had an increased urinary excretion of podocytes [10]. Two available studies on the expression of nephrin in IgAN revealed conflicting data. Gagliardini and co-workers [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 co-workers [12] found a reduction in nephrin and a shift of the podocyte-staining pattern only in IgAN patients with nephrotic syndrome, but not in non-nephrotic IgAN patients.

Most recently, Wang and co-workers [13] reported that serum IgA1 from IgAN patients directly induces apoptosis in podocytes. The findings are most intriguing as the known IgA receptor has never been documented in podocytes. We have demonstrated that pIgA from IgAN patients does not activate podocytes directly, and podocytes lack the known IgA receptor that binds IgA [7]. Our histological demonstration of distinctly separate immunostaining of IgA and nephrin with no co-localization further confirms no direct binding of IgA to podocytes. Despite the less severe histological grading, a reduction in podocyte markers (nephrin and ezrin) is clearly evident in our patients with mild IgAN. Nephrin is the major podocyte specific marker and has a crucial role in the filtration barrier of the glomerular podocyte. Podocin serves as a scaffolding molecule to localize nephrin while synaptopodin is associated with the cytoskeleton of parietal podocytes. Ezrin is a glomerular epithelial cell marker of podocyte injury (podocytopathy) and may help in the histological qualification of diffuse mesangial proliferation and focal segmental glomerulosclerosis. The increased permeability of the filtration barrier in steroid-resistant and proteinuric glomerulopathies may be a consequence of subcellular changes in podocyte-associated proteins following decreased expression of ezrin [14]. Our histochemical findings on nephrin and ezrin are in accord with a recent report of reduced glomerular epithelial protein 1 (GLEPP1) expression in IgAN [15]. GLEPP1, a podocyte receptor membrane protein tyrosine phosphatase located on the apical cell membrane of visceral glomerular epithelial cell and foot processes, is a marker of acute podocyte injury previously reported in puromycin aminonucleoside nephrosis. The absent alteration of SPARC in IgAN may not be totally surprising. SPARC has a putative role for wound repair [16]. In chronic allograft nephropathy, SPARC is speculated to function as an accessory molecule in chronic PDGF-mediated sclerosing interstitial and vascular injury [17]. Neither repair process nor PDGF-mediated podocyte injury is evident in our patients with IgAN.

The next question is how podocyte dysfunction is mediated in IgAN. In this study, we examined the pathophysiological effect of the mediator released from mesangial cells triggered by IgA deposition on podocytes using an in vitro system that we had previously used for studying the TEC function in IgAN [4]. Based on our demonstration of the existence of a glomerulo-tubular cross-talk in IgAN [2], we hypothesize that a similar glomerulopodocytic cross-talk exists in IgAN. To confirm our hypothesis, we conducted podocyte culture experiments using the conditioned medium from HMC pre-incubated...
with different IgA preparations. This medium transfer setting allowed no direct cell–cell communication but simulated the in vivo glomerulo-podocytic communication via humoral factors. The conditioned medium from HMC incubated with plgA from patients with IgAN, but not with plgA from healthy controls, down-regulated the expression of nephrin and ezrin. Activated mesangial cells produce cytokines and chemokines including IL-1, IL-6, TNF-α, MCP-1, TGF-β and PDGF. 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 TEC to produce other pro-inflammatory cytokines and chemokines that eventually lead to tubular damage, interstitial mononuclear cells infiltration and fibrosis [2]. TNF-α and TGF-β produced by HMC following stimulation by plgA from IgAN patients lead to down-regulation of podocyte markers, based on our culture experiments using different neutralizing antibodies. Culturing podocytes with recombinant TNF-α or TGF-β at a concentration comparable to that in the diluted IgA–HMC conditioned medium resulted in similar down-regulation of these podocyte markers. Furthermore, these down-regulatory changes can be abolished with specific neutralizing antibodies, suggesting that both TNF-α and TGF-β play a unique and crucial role in mediating the inflammatory injury along the glomerulo-podocyte axis in IgAN.

Finally, how should we interpret the down-regulation of these podocyte markers in IgAN? Both ezrin and GLEPP1 are markers of acute podocyte injury while nephrin and podocin maintain an intact glomerular filtration barrier. Such down-regulation will more likely lead to functional alteration in glomerular permeability and structural alteration with foot process effacement. No doubt, the podocyte injury will contribute to proteinuria although heavy proteinuria is not a salient feature of IgAN, especially in patients with mild histological severity. Despite increasing knowledge of the molecular composition of the glomerular filtration, the relationship between proteinuria and foot process effacement is unclear. van den Berg and co-workers [18] found no correlation between foot process effacement and proteinuria, suggesting that a different mechanism of podocyte injury may operate in IgAN as compared with other proteinuric glomerulopathies. A pivotal role of cathepsin L and its substrate, the GTPase dynamin, in the induction of proteinuria and associated foot process effacement has recently been suggested [19]. However, our demonstration of a good correlation between the reduction of nephrin expression and proteinuria or a fall in glomerular filtration rate strongly supports the pathologic importance of podocytic injury in IgAN. We speculate that down-regulation of podocyte markers by TNF-α or TGF-β released by mesangial cells following IgA deposition alters the glomerular permeability. This will facilitate mesangial-derived humoral factors/mediators to reach the tubulointerstitium that eventually leads to tubular damage, interstitial mononuclear cells infiltration and fibrosis. The exact mechanism by which these podocyte proteins are down-regulated in IgAN has not been examined but transscriptional suppression of nephrin by TNF-α via activation of the phosphatidylinositol-3-kinase/Akt pathway is a likely possibility [20].

In conclusion, our finding suggests that podocyte markers are reduced in IgAN. The in vitro study implicates that humoral factors (predominantly TNF-α and TGF-β) released from mesangial cells are likely to alter the glomerular permeability in the event of proteinuria and tubulointerstitial injury in IgAN.

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