Combined blockade of angiotensin II and prorenin receptors ameliorates podocytic apoptosis induced by IgA-activated mesangial cells

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Abstract Glomerulo-podocytic communication plays an important role in the podocytic injury in IgA nephropathy (IgAN). In this study, we examine the role of podocytic angiotensin II receptor subtype 1 (AT1R) and prorenin receptor (PRR) in podocytic apoptosis in IgAN. Polymeric IgA (pIgA) was isolated from patients with IgAN and healthy controls. Conditioned media were prepared from growth arrested human mesangial cells (HMC) incubated with pIgA from patients with IgAN (IgA-HMC media) or healthy controls (Ctl-HMC media). A human podocyte cell line was used as a model to examine the regulation of the expression of AT1R, PRR, TNF-α and CTGF by IgA-HMC media. Podocytic nephrin expression, annexin V binding and caspase 3 activity were used as the functional readout of podocytic apoptosis. IgA-HMC media had no effect on AngII release by podocytes. IgA-HMC media significantly up-regulated the expression of AT1R and PRR, down-regulated nephrin expression and induced apoptosis in podocytes. Mono-blockade of AT1R, PRR, TNF-α or CTGF partially reduced podocytic apoptosis. IgA-HMC media activated NFκB, notch1 and HEY1 expression by podocytes and dual blockade of AT1R with PRR, or anti-TNF-α with anti-CTGF, effectively rescued the podocytic apoptosis induced by IgA-HMC media. Our data suggests that pIgA-activated HMC up-regulates the expression of AT1R and PRR expression by podocytes and the associated activation of NFκB and notch signalling pathways play an essential role in the podocytic apoptosis induced by glomerulo-podocytic communication in IgAN. Simultaneously targeting the AT1R and PRR could be a potential therapeutic option to reduce the podocytic injury in IgAN.

Keywords Apoptosis • IgAN • Podocytes • AT1R • Prorenin receptor • Notch1 • NFκB

Introduction

Immunoglobulin A nephropathy (IgAN) remains one of the leading causes of renal failure in many parts of the world. It runs a relentless clinical course with end-stage renal failure in 35–40% of patients after 25–30 years’ follow-up [1]. The disease is characterized by mesangial deposition of polymeric IgA1 (pIgA) [2], followed by proliferation of mesangial cells, increased synthesis of extracellular matrix and infiltration of immune cells [3]. The absence of known IgA receptors in podocytes and renal tubular epithelial cells (TEC) and the lack of IgA binding to these cells support the pathologic finding that IgA is only deposited in the mesangium in IgAN [4, 5]. The most important question is how mesangial IgA deposits lead to damage of other components of the nephron and, ultimately, lead to renal failure.

We identify a novel mechanism involving a glomerulo-tubular crosstalk in the development of tubulointerstitial damage in IgAN [5]. Mediators released from mesangial cells after IgA deposition activate TEC and cause...
subsequent inflammatory changes in the renal interstitium. Podocytes are positioned strategically along the glomerulotubular axis, yet their role in mediating the glomerulotubular crosstalk in IgAN has not been addressed. We have previously demonstrated that podocytes play a contributory role in the development of interstitial damage in IgAN by amplifying the activation of TEC with enhanced TNF-α synthesis following inflammatory changes of HMC [4]. We have also shown that humoral factors, predominantly as tumour necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β) released by HMC following IgA deposition down-regulated the expression of nephrin and ezrin in cultured podocytes [6]. Alteration of these podocytic membrane proteins affects the function of podocytes in maintaining the integrity of the glomerular filtration barrier and leads to heavy proteinuria, glomerulosclerosis, and loss of kidney function [7, 8]. Furthermore, persistence of podocyte injury may activate cascades of cellular and immune processes that lead to irreversible cellular damage, which induces podocyte cell death or the detachment of podocytes from the GBM, and ultimately leads to glomerulosclerosis and kidney failure.

Podocytes have recently been shown to fully express the renin-angiotensin system (RAS) [9]. Mesangial cells release angiotensin II (AngII) following IgA deposition in IgAN, however the impacts of mesangial derived AngII and other humoral factors on the podocytic RAS components largely remain unknown.

The Notch signalling pathway activation plays an important role in inducing apoptosis in podocyte [10]. Activation of the notch pathway leads to the release of the active Notch intracellular domain (ICN). Nucleus translocation of ICN triggers the transcription of classical target genes including the hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1). Animal studies have shown that conditional expression of active Notch1 protein leads to extensive albuminuria, glomerulosclerosis and ultimately renal failure and death [11].

In the present study, we examine how the humoral factors after mesangial IgA deposition alter podocytic RAS and delineate the relationship between the RAS, apoptosis and notch signalling pathway activation within podocytes in IgAN.

Methods

Reagents

Jacalin agarose was purchased from Pierce (Rockford, IL, USA). Roswell Park Memorial Institute Medium (RPMI 1640 medium), fetal bovine serum (FBS) and recombinant connective tissue growth factor (CTGF) were obtained from Life Technologies (Rockville, MD, USA). F(ab′)2 fragment of FITC-conjugated rabbit anti-human IgA and FITC-conjugated pre-immune rabbit antibodies were obtained from Dako (Kyoto, Japan). Neutralizing antibodies to TNF-α, TNF-α receptor 1 and 2 (TNFR1 and TNFR2), ELISA kits for TNF-α were obtained from R&D Systems (Minneapolis, MN, USA). ELISA kit for CTGF was from MyBioSource (San Diego, CA, USA). Nephrin antibody was obtained from Fitzgerald Industries International (Concord, MA, USA). Rabbit polyclonal anti-AngII receptor subtype-I (AT1R) and AngII receptor subtype-II (AT2R) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin antibody was obtained from Lab Vision Co. (Fremont, CA, USA). Antibodies to prorenin receptor (PRR), notch1, HEY1, neutralizing anti-human CTGF and horseradish peroxidase (HRP)-conjugated rabbit anti-guinea pig IgG were obtained from Abcam (Cambridge, UK). Secondary antibodies for immunoblotting were obtained from Dako (Kyoto, Japan). All other chemicals 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 controls) gave their written informed consent for blood collection or kidney biopsy. Venous blood was collected from 18 Chinese patients (10 males and 8 females) with clinical and renal immunopathological diagnosis of primary IgAN. 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. Thirty milliliters of blood was collected from each patient at clinical quiescence. The sera were isolated and frozen at −70 °C until isolation of IgA. Twenty healthy subjects (10 males and 10 females), comparable in age and race, with no microscopic haematuria or proteinuria, were recruited as healthy controls. Sera were similarly collected from these individuals for processing.

Cell culture and isolation of pIgA

Isolation, characterization and culture of HMC were performed as previously described [12]. Briefly, glomeruli

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were prepared from the cortex of human cadaveric kidney judged to be unsuitable for transplantation or from the intact pole of kidneys removed for circumscribed tumor. Histological examination of these kidney samples revealed no renal pathology. Isolated HMC were grown in RPMI 1640 medium supplemented with glutamine (2 mmol/l), N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES) (10 mmol/l), penicillin (50 U/ml), streptomycin (50 mg/ml), and 20 % fetal calf serum in an atmosphere of 5 % CO2–95 % air. A conditionally immortalized human podocyte cell line is established by transfection with a temperature-sensitive SV40-T gene and was obtained from University of Bristol [13]. 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 podocytes including nephrin, podocin, CD2AP, synaptopodin, P-cadherin and ZO-1. In all experiments, HMC between passages 3rd to 6th and differentiated podocytes between passages 12th and 17th were used and were growth arrested with culture media containing 0.5 % FBS unless otherwise stated.

Preparation of conditioned media

Conditioned media were prepared by incubating HMC with the media containing plgA (a total of ten plgA preparations were prepared: five plgA preparations were from IgAN patients with each preparation containing pooled plgA isolated from three different IgAN patients; the other five plgA preparations were from healthy controls with each preparation containing pooled plgA isolated from four different healthy controls) isolated from IgAN patients (IgA-HMC media) or healthy controls (Ctl-HMC media) at a final concentration 50 μg/ml for 48 h. The use of IgA-HMC media was based on our findings that plgA from IgAN patients does not activate podocytes directly, and the absence of known IgA receptor in podocytes that binds IgA [4]. 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 [14]. IgA-HMC and Ctl-HMC media were collected and stored at −70 °C until use. Conditioned media collected from HMC cultured without the addition of IgA were used as plain media control in all experiments. HMC conditioned media were diluted 2-, 8- and 32-fold for dose-dependent experiments and eightfold for all other experiments with RPMI 1640 medium containing 0.5 % FBS unless otherwise stated.

Flow cytometry

Human mesangial cells or podocytes were grown to log phase and harvested by incubation with 0.05 % trypsin/0.02 % EDTA for 5 min at room temperature. The cells were adjusted to 5 × 10⁶ per ml and 200 μl of cell suspension were used in binding assays. Staining was performed at 4 °C with staining buffer consists of phosphate-buffered saline (PBS) with 1 % FBS and 0.1 % sodium azide. The cells were incubated with 100 μl of plgA (final concentration 50 μg/ml) for 30 min. 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 analyzed using a Coulter Cell Lab QUANTA (Miami, Fl, USA). A minimum of 5000 cells for each sample was analyzed. Fluorescence intensity was evaluated by comparing the mean fluorescence channels. The result was expressed as mean fluorescence intensity (MFI).

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Podocytic CTGF, TGF-β or AngII release

Growth arrested podocytes were cultured in six-well culture plate (1 × 10⁶ cells per well) with different dilutions of conditioned media or recombinant TNF-α (50 pg/ml) or CTGF (1 μg/ml) for 48 h. After culture, culture supernatants were collected for the determination of TNF-α, CTGF or AngII concentration. AngII was measured by an enzyme immunoassay using an anti-AngII Fab’ monoclonal antibody labeled with acetylcholinesterase (SPI bio; Massy Cedex, France). The minimum detectable concentration was 1 pg/ml and the intra-assay coefficient of variation was 7 %. TNF-α or CTGF levels were measured with ELISA kits (R&D Systems or MyBioSource). The detection sensitivity for TNF-α or CTGF was 5 or 100 pg/ml, with the intra-batch coefficient of variation of ±7.8 or 8.4 % respectively.

Knockdown of PRR expression in podocytes by RNA interference

Human PRR mRNA was specifically knockdown using a commercially available RNA interference (siRNA) oligonucleotides (Dharmacon, Lafayette, CO, USA). Before transfection, human podocytes were maintained in RPMI 1640 medium with 10 % FBS and then transfected with DharmaFECT (Dharmacon) and the human PRR siRNA (ATP6AP2; Dharmacon; 100 nmol/l) according to the manufacturer’s instructions. The gene knockdown efficiency was confirmed by data from quantitative RT-PCR.
and western blotting (Supplementary Fig. S1). Scrambled non-targeting siRNA control was used as control. For experiments involving PRR mRNA knockdown, podocytes were transfected with siRNA at 48 h before beginning an experiment.

Effects of HMC conditioned media on podocytes

To examine the effect of HMC conditioned media on the expression of AT1R, PRR, and release of AngII, TNF-α or CTGF in podocytes, growth arrested podocytes were seeded onto six-well culture plate (1 × 10^6 cells per well) and were cultured with different dilutions of HMC conditioned media (32-, 8- and 2-fold diluted) for either 6 h (for RT-PCR) or 48 h (for western blotting). For investigation the effects of HMC conditioned media on the expression of neprhin and apoptotic response in podocytes, growth arrested podocytes were cultured with eightfold diluted HMC conditioned media, exogenous TNF-α (50 pg/ml) or CTGF (0.1 µg/ml) for either 6 h (for RT-PCR) or 48 h (for apoptosis assay). After culture, culture supernatants or cells were collected for ELISA, total RNA isolation, cell lysate apoptosis assay (2015) 20:907–920

Quantification of AT1R, PRR and nephrin mRNA expression by real-time PCR

Real-time PCR was performed as previously described [12]. Primer sequences and gene bank accession numbers are listed in the Supplementary Table S1. Real-time PCR amplification was performed in ABI Prism 7500 Sequence Detection system using the SYBR-Green reaction kit (Applied Biosystems; Foster City, CA, USA). The data obtained were analyzed using the comparative CT (cycle threshold) method.

Quantification of AT1R, PRR, nephrin, activated notch1 and HEY1 protein expression by western blot analysis

Cell extracts were pelleted at 15,000 g. Ten micrograms of total protein extract from 10^6 cells were electrophoresed and transferred to polyvinylidene difluoride membranes. The membrane was probed with anti-actin, anti-AT1R, anti-PRR, anti-nephrin, anti-activated notch1, or anti-HEY1 (all at 10 µg/ml) in phosphate-buffered saline–Tween for 16 h at 4 °C. The membrane was incubated with a peroxidase-labeled secondary antibodies and the antigen–antibody reaction was detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). For semi-quantitative determination of protein expression, western blotting images were detected with Chemidoc Imaging System (BioRad) and the density of the bands was quantitated using the ImageQuant software (Molecular Dynamic, Sunnyvale, CA, USA). Densitometry results were reported as arbitrary integrated values after normalization with the values of the actin signal.

Immunohistochemical analysis

Renal cortical tissues were obtained from normotensive IgAN patients (eight from patients with proteinuria <1 g/day and another eight from patients with proteinuria >1 g/day). They had not previously received angiotensin-converting enzyme inhibitor or AT1R antagonist. Control renal tissues were obtained from the intact pole of kidneys removed for single circumscribed tumor in seven normotensive subjects. Paraffin-embedded renal tissues were sectioned at a thickness of 5 µm. The sections were deparaffinized with xylene and then rehydrated through a descending gradient of ethanol. Glomerular expression of TNF-α, TNFR1, AT1R, PRR and CTGF were detected by immunoperoxidase staining using specific rabbit or mouse antibodies (rabbit anti-TNF-α, TNFR1, AT1R, PRR and CTGF from Abcam; mouse anti-PRR from Sigma; final dilution at 10 µg/ml). The bound rabbit or mouse antibodies were visualized in brown colour using the Dako Envision Plus System (Dako). Apoptosis was determined by TUNEL.
(terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining carried out with the ApoTaq Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA) following the manufacturer’s protocol. Cells were regarded as apoptotic if they exhibited brown stained nuclei.

**Statistical analysis**

All data (cell culture experiments) were expressed as mean ± standard deviation (SD). Inter-group differences for continuous variables were assessed by the unpaired t test. The protein synthesis in podocytes following exposure to different concentrations of conditioned media was analyzed with multivariate ANOVA followed by Bonferroni’s method to control for multiple testing. All p values quoted are two-tailed and significance is defined as p < 0.05.

**Results**

**IgA-HMC-media up-regulate podocytic TNF-α and CTGF, but not AngII release**

The binding of plgA prepared from IgAN patients was significantly higher than that from the healthy controls (MFI 10.9 ± 0.40 vs. 6.44 ± 0.26; p < 0.001; Fig. 1a). As there was no binding of the plgA prepared from patients or control to podocytes, we tested the effects of IgA-HMC media on podocytic release of TNF-α, CTGF, and AngII, which have been demonstrated as the major humoral effectors in the development of podocytic abnormality in IgAN. There was no increase in mRNA expression for angiotensinogen or AngII release in podocytes cultured with IgA-HMC media compared with Ctl-HMC media (Fig. 1b). The AngII concentration in eightfold diluted IgA-HMC media is 0.91 ± 0.42 pg/ml. As shown in Fig. 1b, the AngII concentrations in culture media harvested from podocyte cultured with different dilution of IgA-HMC-conditioned media are all greater than 2 pg/ml. This may represent the basal concentration of AngII released by cultured podocytes but are not affected in the presence of IgA-HMC media. There were dose dependent up-regulation of the mRNA expression or protein release of TNF-α and CTGF when podocytes were incubated with IgA-HMC media from IgAN patients when compared with Ctl-HMC media (Fig. 1c, d). As the TNF-α and CTGF concentration in eightfold diluted IgA-HMC media are 48.17 ± 5.32 and 567.32 ± 89.81 pg/ml respectively, suggesting that podocytes cultured with IgA-HMC media release more TNF-α and CTGF.

**Fig. 1** (a) Flow cytometry assay of IgA binding to podocytes and HMC. Binding of plgA from IgAN patients (plgA-IgAN; n = 18) to HMC but not to podocyte were increased when compared with healthy controls (plgA-Ctl; n = 20). The binding assay was performed using same amount of plgA (50 μg/ml) and the equal number of cells (1 × 10^6 cells). The data represent the mean ± standard deviation of the mean fluorescent intensity (MFI). *p < 0.05 versus Ctl. Histograms (b)–(d) showing the mRNA expression of angiotensinogen and AngII synthesis, mRNA expression or protein release of TNF-α and CTGF by podocytes cultured with IgA-HMC media (black bar) or Ctl-HMC media (white bar). *p < 0.05 versus plain media control. The results represent the mean ± standard deviation from five individual experiments.
IgA-HMC-media down-regulate nephrin expression and induce apoptosis in podocytes

There were significant increases in percentage of apoptotic cells (defined as relative % of annexin V+ and PI− cells) (7.76 ± 0.47 vs. 1.30 ± 0.34 %, p < 0.01) and caspase 3 activity (6.11 ± 0.22 vs. 1.13 ± 0.10, p < 0.01) when podocytes were incubated with IgA-HMC media when compared with Ctl-HMC media (Fig. 2a, b). Increased apoptotic cell % and caspase 3 activity were also observed when podocytes were cultured with exogenous recombinant TNF-α or CTGF. The expression of nephrin mRNA or protein were significantly reduced when podocytes were incubated with exogenous TNF-α, CTGF, or IgA-HMC media (Fig. 2c, d).

Expression of components of the renin-angiotensin system (RAS) in podocytes incubation with IgA-HMC media

Podocytes express a functional intrinsic renin-angiotensin system (RAS), which may play an important role in the mesangial-podocytic communication in IgAN. We examined the expression of essential components of the RAS in podocytes cultured with IgA-HMC media (Table 1). Although there were no changes in AngII release or the expression of enzymes that controlling the formation or degradation of AngII, there were significant increases in the expression levels of AT1R (2.72 ± 0.32 vs. 1.25 ± 0.30, p < 0.05) and PRR (3.19 ± 0.38 vs. 1.52 ± 0.42, p < 0.05) in podocytes incubated with IgA-HMC media when compared with Ctl-HMC media (Table 1).

IgA-HMC media up-regulate AT1R and PRR expression in podocytes

To further validate the qPCR screening results, which demonstrated the AT1R and PRR mRNA up-regulation by IgA-HMC media, experiments were designed to examine the dose dependent effects of IgA-HMC media on mRNA and protein expression of AT1R and PRR by podocytes. The expression of AT1R in podocytes was up-regulated by incubation with IgA-HMC media (eight or twofold diluted) (Fig. 3a, b). This up-regulated expression of AT1R was also observed when podocytes were cultured with exogenous recombinant TNF-α, but not CTGF. Although podocytic expression of PRR was significantly increased as determined by quantitative RT-PCR and western blotting respectively. Concentrations of TNF-α and CTGF used: 50 pg/ml and 0.1 μg/ml. Podocytes incubated with 0.25 μg/ml doxorubicin hydrochloride (ADR) was used as positive control in these experiments. *p < 0.01 versus plain media control (Media Ctl). The results represent the mean ± standard deviation from five individual experiments.
by incubation with IgA-HMC media (eight or twofold diluted), the PRR expression was not affected by incubation with exogenous recombinant TNF-α or CTGF (Fig. 3c, d).

**Effect of blockade of RAS, NFκB, TNF-α receptors on TNF-α or CTGF release by podocytes cultured with IgA-HMC media**

We have previously shown the important roles of RAS, NFκB and TNF-α receptors in the pathogenesis of IgAN, we herein examined whether blockade of these elements reduces the TNF-α or CTGF synthesis by podocytes. Incubation with angiotensin-converting enzyme inhibitor (ACEi), blockade of AT1R, PRR, TNFR2, NFκB, and CTGF neutralization did not alter the TNF-α release by podocytes incubated with IgA-HMC media (Fig. 4a). Blockade of TNFR1 or TNF-α neutralization ameliorated but not abolished the TNF-α release by podocytes, suggesting the autocrine mechanism through TNFR1 contributed partly on the TNF-α release by podocytes incubated with IgA-HMC media. Suppression but not abolishment of CTGF release by podocytes incubated with IgA-HMC media was achieved by incubation with ACEi, blockade of AT1R, PRR, TNFR1, NFκB, CTGF, or TNF-α neutralization whereas TNFR2 blockade has no effect (Fig. 4b).

**Effect of blockade of RAS, TNF-α receptors on AT1R or PRR expression by podocytes cultured with IgA-HMC media**

ACEi incubation, blockade of AT1R, PRR, TNFR2, or CTGF neutralization did not affect the AT1R expression by podocytes incubated with IgA-HMC media (Fig. 5a). Blockade of TNFR1, TNF-α neutralization, or incubation with cell permeable NFκB inhibitor significantly abolished the up-regulated AT1R expression by podocytes. The up-regulated expression of podocytic PRR cultured with IgA-HMC media was only abolished by blockade with PRR siRNA (Fig. 5b). ACEi incubation, blockade of AT1R, PRR, TNFR1, TNF2, TNF-α or CTGF neutralization have no effect on the PRR expression by podocytes incubated with IgA-HMC media.

**Effect of blockade of RAS, TNF-α receptors on caspase 3 activity and nephrin expression by podocytes cultured with IgA-HMC media**

Blockade of TNFR2 did not affect the increased caspase 3 activity (Fig. 6a) or reduced nephrin expression (Fig. 6b) by podocytes incubated with IgA-HMC media. Blockade of AT1R, TNFR1, NFκB, PRR, TNF-α or CTGF neutralization only partially ameliorated the up-regulated caspase 3 activity and reduced nephrin expression. Complete reduction of the increased caspase 3 activity or the restoration of nephrin expression were only achieved by simultaneous TNF-α and CTGF neutralization or PRR blockade together with blockade of AT1R, NFκB inhibition, or TNF-α neutralization.

**Activation of the podocytic NFκB and notch1 signals by IgA-HMC media**

Incubation with IgA-HMC media induced activation of the NFκB, notch1 and HEY1 expression by podocytes (Fig. 7a–c). Incubation with exogenous recombinant TNF-

| Table 1 Expression of RAS components in podocytes |
|---------------------------------|----------------|-----------------|-----------------|
| Gene               | Plain media | Ctl-HMC media  | IgA-HMC media  |
| Renin              | 2.07 ± 1.70 | 2.76 ± 1.59    | 2.65 ± 1.37    |
| PRR                | 1.55 ± 0.71 | 1.52 ± 0.42    | 3.19 ± 0.38*   |
| ACE                | 1.53 ± 0.67 | 1.58 ± 0.68    | 1.56 ± 0.91    |
| ACE2               | 2.59 ± 1.46 | 2.51 ± 1.77    | 2.90 ± 1.94    |
| Angiotensinogen    | 1.50 ± 0.71 | 1.55 ± 0.74    | 1.44 ± 0.64    |
| AT1R               | 1.09 ± 0.64 | 1.25 ± 0.30    | 2.72 ± 0.32*   |
| AT2R               | 1.02 ± 0.21 | 1.05 ± 0.24    | 1.07 ± 0.19    |
| Neprilysin         | 1.41 ± 0.72 | 1.39 ± 0.57    | 1.33 ± 0.65    |
| Cathepsin G        | 1.40 ± 0.59 | 1.68 ± 0.81    | 1.55 ± 0.77    |
| Aminopeptidase A   | 1.52 ± 0.40 | 1.44 ± 0.66    | 1.50 ± 0.46    |
| Tissue Kallikrein  | 1.13 ± 0.45 | 1.22 ± 0.79    | 1.12 ± 1.02    |
| Chymase            | 2.02 ± 0.95 | 2.46 ± 1.41    | 2.06 ± 1.44    |

All results are expressed as fold change of gene expression compared to podocytes cultured in growth-arrested media. The results represent the mean ± standard deviation from five individual experiments.

* _p < 0.05 versus plain media*

@ _p < 0.05 versus Ctl-HMC media*
α induced activation of the podocytic NFκB but not notch1 or HEY1. On the contrary, incubation with exogenous recombinant CTGF induced activation of the podocytic notch1 and HEY1, but not NFκB. NFκB inhibitor, but not the γ-secretase blocker LY450139, abolished the activation of podocytic NFκB by IgA-HMC media or TNF-α (Fig. 7a). Incubation with LY450139, but not SN50, eliminated the activation of podocytic notch1 and HEY1 by IgA-HMC media or CTGF (Fig. 7b, c).

**Dual blockade of the podocytic NFκB and notch1 activation rescues nephrin expression and prevents apoptosis**

Blockade with either SN50 or LY450139 incompletely restored the reduced nephrin expression, partially ameliorated the increased % of apoptotic cell and caspase 3 activity by podocyte incubated with IgA-HMC media (Fig. 8a–c). Simultaneous incubation with SN50 and LY450139 was essential to achieve complete prevention of these apoptotic events and restoration of the reduced podocytic nephrin expression induced by IgA-HMC media.

**Increased glomerular apoptosis and expression of TNF-α, TNFR1, AT1R, PRR and CTGF in IgAN patients**

Up-regulated glomerular expression of TNF-α, TNFR1, AT1R, PRR and CTGF was observed in IgAN patients when compared to normal subjects (Fig. 9). Increased number of glomerular apoptotic cells was also visualized in IgAN patients. Of particular note, these up-regulated glomerular TNF-α, TNFR1, AT1R, PRR or CTGF expression and apoptotic events were much more profound in IgAN patients when proteinuria exceeded values of 1 g/day than those with proteinuria of less than 1 g/day.

**Discussion**

In this study, we have confirmed our previously observation that although podocytes do not bind pathogenic pIgA, humoral factors released following pIgA binding to mesangial cells activated synthesis of TNF-α by podocytes. We have extended our previously findings that other than
TNF-α, mesangial-tubular crosstalk triggered by deposited IgA activates the synthesis of CTGF; these two key effectors were able to promote apoptosis in podocytes. We have further demonstrated that mesangial-tubular crosstalk triggered by mesangial IgA deposition induced the overexpression of AT1R and PRR in podocytes, and these two receptors utilize different signal mechanisms contributing to the podocytic apoptosis.

Under normal physiological condition, podocytes express a functional intrinsic RAS characterized by enzymatic activities predominantly leading to ANG-(1–7) and ANG-(1–9) formation, and AngII degradation. ANG-(1–9) counteracts the proinflammatory actions of AngII. It has been suggested that podocytes play a specific role in the maintenance of intraglomerular RAS balance [9]. Nonetheless, these enzymatic activities are altered in non-physiological environment such as hyperglycemia that mimics diabetic kidney disease [9, 15]. We have shown here that neither AngII release, nor the expression of enzymes that control the formation or degradation of AngII, changes in podocytes following IgA-HMC media incubation. However, there were significant increases in the expression level of AT1R and PRR. We have previously documented a glomerular-tubular crosstalk triggered by mesangial IgA deposition activates the up-regulation of AT1R in TEC and leads to the tubulointerstitial damage [16]. A similar up-regulation of AT1R associated with mesangial IgA deposition was observed in the current study, and this up-regulated AT1R expression is controlled by podocytic TNF-α/TNFRI axis through signal...
from NFκB activation. Despite a link between TNFR1 activation by TNF-α and AT1R up-regulation has not yet been reported in podocytes, data generated from cardiac fibroblasts culture depicted that TNF-α up-regulates AT1R density and enhance the pro-fibrotic effect of AngII [17]. In addition, activation of NFκB is essential for the AT1R up-regulation by TNF-α in cardiac fibroblast [18]. That piece of evidence from cardiac fibroblast may provide intriguing possibility that similar mechanism may take place on podocytes, which deserves further investigation.

The renin-angiotensin system plays an indisputable role in the pathogenesis of kidney disease. A distinctive RAS presents in different resident kidney cells [19, 20]. AngII
induces apoptosis in rat podocytes with up-regulation of the expression of Fas, FasL, and Bax and down-regulation of Bcl-2 expression [21]. Hypertensive rats induced by continuous AngII infusion show podocytic apoptosis associated with decreased nephrin expression [22]. The slit diaphragm protein nephrin play a pivotal role in preventing passage of protein through the glomerular barrier [23]. In human study, a marked reduction of nephrin mRNA and extracellular nephrin was detected in IgAN but not in minimal change nephropathy (MCN) or focal segmental glomerulosclerosis (FSGS) [24]. Podocyte loss is associated with the increased disease severity in IgAN and contributes to the progressive glomerular sclerosis and filtration failure [25]. Consistent with these results, our current data demonstrated IgA-HMC media induced apoptosis and reduced nephrin expression by podocyte. Unlike the results obtained from rodent experiments, our data from human podocytes culture showed that the

Fig. 8 Dual blockade of the podocytic NFκB and Notch1 activation rescues nephrin expression and prevents apoptosis. Effects of inhibition of NFκB or/and notch1 on (a) apoptosis, (b) caspase 3 activity or (c) nephrin expression in podocytes following incubation with IgA-HMC media. Combined blockade of the NFκB and notch signals also significantly reduced the TNF-α or CTGF associated apoptotic events and nephrin reduction. Concentrations of TNF-α and CTGF used: 50 pg/ml and 0.1 μg/ml. @ or * signifies p < 0.05 or <0.01 versus podocytes incubated with IgA-HMC media without intervention. The results represent the mean ± standard deviation from five individual experiments. SN50: peptide inhibits the translocation of the active NF-κB complex into the nucleus; LY450139: γ-Secretase or the notch receptor blocker

Fig. 9 Localization of TNF-α, TNFR1, AT1R, PRR, CTGF expression and apoptotic cell in renal biopsies from normal subjects and IgAN patients. Representative immunohistochemical staining of TNF-α, TNFR1, AT1R, PRR and CTGF expression in glomeruli from normal subjects and IgAN patients. Compared to that of the normal subjects (Ctl; left column), increased apoptotic cells and expression of TNF-α, TNFR1, AT1R, PRR and CTGF was observed in glomeruli from IgAN patients with proteinuria >1 g/day (right column). These up-regulated expressions were much less obvious from IgAN patients with proteinuria <1 g/day (middle column)
podocytic apoptosis was independent of AngII up-regulation, but associated with increased AT1R expression and the activation of TNFR1/TNF-α axis. Our present data from the blockade experiments have suggested that the upregulated expression of AT1R in podocytes is only partially responsible for the apoptotic response towards activation by IgA-HMC media, some additional mechanisms must therefore be involved.

The prorenin receptor is an exciting addition to the RAS [26]. The binding of prorenin to the prorenin receptor not only causes a non-proteolytic activation of prorenin leading to the activation of the RAS, but also stimulates the receptor’s own intracellular signaling pathways independent of the RAS. Within the kidney, PRR is present in the glomerular mesangium and podocytes, which play an important role in the maintenance of the glomerular filtration barrier. Angiotensin-independent effects of prorenin activation have been reported. Renin and prorenin induced TGF-β release in mesangial cells in the presence of renin inhibitors, ACE inhibitors and/or AT1 receptor antagonists [27, 28]. Interestingly, our present data have shown that other than AT1R, expression of PRR was also up-regulated by IgA-HMC media. This up-regulated PRR expression elicited the downstream events including increased CTGF synthesis and podocytic apoptosis. However, the upregulated podocytic CTGF synthesis and apoptosis by IgA-HMC media was not solely PRR dependent, the AT1R upregulation by IgA-HMC media also contributed noticeably to these processes as described earlier. CTGF is a crucial effector for the development of diabetic glomerulosclerosis [29]. CTGF is mainly expressed in podocytes and its expression is markedly up-regulated and associated with fibrotic lesions [30]. Overexpression of CTGF in murine podocytes exacerbates proteinuria and through a functional impairment and loss of podocytes [31]. High glucose stimulates mesangial cells to secrete TGF-β, which is stored on cell surface of podocytes as latent inactive form. The latent TGF-β is activated by AngII dependent process to induce podocytic CTGF synthesis and contributes to the podocytic apoptosis [32]. In rat mesangial cells, high glucose increases expression of PRR, and leads to the enhanced production of CTGF [33, 34]. We have previously demonstrated that IgA from IgAN patient increased TGF-β production by HMC via increased AngII [35]. In the present study, we observed the up-regulation of podocytic PRR, CTGF and apoptosis in podocytes by IgA-HMC media. Taken together, it is logical to postulate that in IgAN, similar mechanism to that in diabetic nephropathy, in which the loss of podocyte involving the activation of the mesangial-podocytic axis through TGF-β-PRR-CTGF is operating.

In this study, we have identified IgA-HMC media induced podocytic apoptosis through the activation of two axes: the TNF-α/TNFFR1/AT1R and the PRR/CTGF. We have further shown that whereas activation of the TNF-α/TNFFR1/AT1R axis was NFκB-dependent, the PRR/CTGF axis utilizes the notch1 signal to induce podocytic apoptosis. Our data are consistent with recent findings from the literature. Podocytic notch signaling pathway plays an important role in glomerular diseases and diabetic nephropathy [10, 36]. Expression of notch1, notch2 and Jagged1 in proteinuric nephropathies including IgAN correlates with the amount of proteinuria [37]. In cultured murine podocytes, transgenic expression of active notch1 induces apoptosis [10]. However, it remains obscure how IgA-HMC media lead to PRR activation by podocytes.

It has been reported that PRR-knockout mice developed nephrotic syndrome, and in vitro PRR knockdown led to altered podocytic cytoskeleton [38]. It should be noted that all the data were obtained with a murine KO model or using an immortalized mouse podocyte cell culture. Different mechanisms may be observed in human podocyte cell line used in the present study and deserves further investigation. Podocyte expression of Bcl-2 has been shown to be up-regulated in early-stage disease and down-regulated in late-stage disease [39]. This finding did not contradict our present observation. Indeed, the same study also have demonstrated that up-regulation of Bcl-2 results in increased expression of p27 and inhibition of cell proliferation, suggesting an anti-proliferative rather than anti-apoptotic role of Bcl-2 over-expression in early-stage of IgAN. Nonetheless, it should be noted that in vitro data may not fully translate to the complicated pathogenesis of podocyte apoptosis observed in IgAN patient.

Our preliminary data on the immunochemical staining of the up-regulated glomeruli expression of TNF-α, TNFR1, AT1R, PRR, CTGF and the increased number of apoptotic cells in IgAN patients provide possible link for the translation of our in vitro results to the bedside. However, there are limitations in the present study. Clinical evidences demonstrating how the dysregulated expression of TNF-α, TNFR1, AT1R, PRR, CTGF lead to podocytic apoptosis in IgAN patients are lacking. Furthermore, it would be very important to test the protective role of dual blockade of AT1R and PRR using an appropriate animal model, and warrants further investigation.

Based on the present results and published data from literature, a schema on the role of mesangial-podocytic communication related to the development of podocytic injury in progressive IgAN is illustrated in Fig. 10. Humoral mediators released from the glomerular mesangium following IgA deposition induced TNF-α synthesis by the podocytes. The released TNF-α may amplify its own production in an autocrine manner. TNF-α up-regulated the expression of podocytic TNF receptors and AT1R through a NFκB dependent mechanism. This TNF-α/TNFFR1/AT1R
axis leads to further apoptosis and nephrin reduction in podocyte. Humoral mediators released from the glomerular mesangium following IgA deposition also lead to enhanced PRR expression and CTGF production which is controlled by the activation of latent TGF-β on the podocyte. This PRR/CTGF axis leads to podocytic apoptosis and nephrin reduction through activation of notch1. Furthermore, AT1R activation also triggers the CTGF production by podocytes and forming a link between the two axes of podocytic apoptosis induction. Dual blockade of activation of NFκB and notch1 is essential to abolish the podocytic damage following mesangial plgA deposition.

In summary, our study revealed that the mesangial-podocytic crosstalk after mesangial pIgA deposition increases the synthesis of TNF-α and CTGF, up-regulates the expression of AT1R and PRR, and induces apoptosis by podocytes. These events are regulated by the NFκB and notch1 dependent signals and dual blockade of these pathways is essential to ameliorate the podocytic injury in IgAN.

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