ORIGINAL RESEARCH

Serum IgA1 from patients with IgA nephropathy up-regulates integrin-linked kinase synthesis and inhibits adhesive capacity in podocytes through indirect pathways

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Abstract

Purpose: To investigate the influence of IgA1 isolated from IgA nephropathy (IgAN) patients on integrin-linked kinase (ILK) synthesis and adhesive capacity of podocytes through indirect pathways.

Methods: IgA1 was isolated from healthy control or IgAN patients' sera using jacalin affinity chromatography and S-200 chromatography. Podocytes were treated with medium from mesangial cells incubated with aggregated IgA1 (aIgA1, 100 µg/ml), in the presence or absence of valsartan (10⁻⁵M) or neutralizing antibodies of tumor necrosis factor-α (TNF-α, 50 ng/ml). Adhesive capacity of podocytes was assessed by cell counting manually and hexosaminidase assay. Real-time PCR and western blotting were used to detect the expression of ILK.

Results: Medium from mesangial cells incubated with aIgA1 from IgAN patients reduced podocyte adhesion to collagen compared with medium from mesangial cells incubated with control medium(RPMI-1640 with 0.5% FBS) (35.0±4.8% vs. 60.0±2.0%; *P*<0.05). While medium from mesangial cells incubated with aIgA1 from IgAN patients upregulated ILK expression in podocytes at mRNA and protein levels compared with medium from mesangial cells

without aIgA1 incubated (1.6-fold and 1.38-fold higher than control, respectively, *P*<0.05). Defects in podocyte adhesion and up-regulation of ILK synthesis induced by medium from mesangial cells incubated with aIgA1 from IgAN patients can be partially reversed by the pre-treatment for 1 hour with valsartan(*P*<0.05), while pre-treatment with neutralizing antibodies of TNF-α produced no protective effect on podocytes(*P*>0.05).

Conclusion: Serum IgA1 from IgAN patients may inhibit adhesive capacity and up-regulate ILK synthesis in podocytes through indirect pathways.

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis in the world, and is characterized by mesangial deposition of polymeric IgA1 (pIgA1) complexes and proliferation of mesangial cells. Recent studies illustrate that deposition of the higher molecular weight forms of undergalactosylated IgA1 in mesangial areas may contribute to pathogenic mechanisms in IgAN.^{1,2} Aggregated

IgA1 complexes, in serum and kidney, play a pivotal role in the development of this disorder due to their ability to bind specifically to mesangial cells and induce production of a number of cytokines, which have been linked to the development and progression of renal injury.³⁻⁶ In-vitro, heat aggregated IgA1 (aIgA1) was also a succedaneum for large-molecular weight IgA1 complexes which can stimulate mesangial cells.^{7, 8}

Although pIgA1 deposition is found predominantly within mesangial regions of glomeruli and is considered the initiating event in the pathogenesis of IgAN, as there is a strong correlation between podocyte injury and the severity of the disease.⁹ Podocyte injury could lead to glomerulosclerosis in IgAN. Two mechanisms have been suggested in the process of podocyte loss: detachment from the glomerular basement membrane (GBM) and apoptosis.¹⁰ Several observations have found that some of the podocytes in the urine of animal models or humans with kidney disease are viable, and can be propagated.^{11, 12} These observations indicate that detachment of podocytes which had poor adhesive capacity may be a major cause of podocytopenia. In fact, loss of cell anchorage to the GBM may worsen proteinuria and glomerulosclerosis. However, the mechanism by which initially intramesangial IgA1 deposits can lead to podocyte loss remains unknown.

Integrin linked kinase (ILK), a ubiquitously expressed protein serine/threonine kinase, appears to be a potential regulator of podocyte adhesive capacity. It plays a key role in integrin-mediated cell-matrix interaction and its overexpression can lead to reduction of podocyte-GBM adhesion. Kretzler et al.^{13,14} reported that up-regulation of ILK in podocytes can lead to an adhesion defect and eventually in glomerular dysfunction. Furthermore, ILK was considered a hinge in the cell-matrix adhesion network. However, the role of ILK in IgAN remains to be elucidated.

Recently, we described a potential mechanism involving mesangial-podocyte communication in the development of podocyte apoptosis in IgAN.¹⁵ In our

present study, we postulate that abnormal IgA1 deposits in the kidney trigger mesangial cells to produce a variety of cytokines and growth factors which pass through GBM by diffusion or other mechanisms, and reduce podocytes adhesion. To support this, an *in-vitro* study was performed to determine the indirect effect of IgA1 on podocytes. We used the IgA-mesangial cell conditioned medium (specially treated medium from mesangial cells incubated with aIgA1 from IgAN patients or healthy controls) to culture the podocytes, and then investigated the changes of adhesive capacity and the synthesis of ILK. Furthermore, we examined the effects of potential mediators (angiotensin II and tumour necrosis factor- α) released by mesangial cells in response to aIgA1 on podocytes.

Materials and methods

Experimental protocol

Three sets of experiments were performed. In experiment #1, IgA1 was isolated from normal human or IgAN patient sera using jacalin affinity chromatography and Sephacryl S-200 molecular sieve chromatography, then monomeric IgA1 (mIgA1) fraction was transformed to aggregated IgA1 (aIgA1) by keeping at 63°C for 150 min. In experiment #2, mesangial cell-conditioned medium was prepared. Mesangial cells were incubated with or without aIgA1 (100 µg/ml) for 48h and then supernatants were harvested and stored at -70 °C until used. Before treating podocytes, the medium from mesangial cells was diluted 10-fold with RPMI 1640 medium (mesangial cell-conditioned medium). In experiment #3, podocytes were treated with the above diluted medium and divided into six groups: PI group, podocytes were exposed to medium from mesangial cells incubated with aIgA1 from IgAN patients; NI group, podocytes were exposed to medium from mesangial cells incubated with aIgA1 from normal control subjects; CI group, podocytes were exposed to medium from mesangial cell incubated in serum-free medium without aIgA1. The PI group was also pretreated for 1 h with valsartan (10-

⁵M, PI+V group) or neutralizing antibodies of TNF- α (50 ng/ml, PI+T group). Podocytes cultured in serum-free RPMI 1640 medium was considered a control group (C group). To exclude the direct effect of residual aIgA1 in mesangial cell-conditioned medium, aIgA1 (10 μ g/ml) was added directly to podocytes incubated in serum-free medium. In the following study, the adhesive capacity of podocytes and expression of ILK were analyzed.

Patients, control subjects and isolation of human IgA1

The study was carried out in accordance with principles of the Declaration of Helsinki and approved by the Medical Ethics Committee of hospital. Written informed consent was obtained from each participant. Twenty-two Chinese patients with primary IgAN confirmed by renal biopsy were studied. All patients were symptomatic for more than 12 months with a 24-hour urinary protein excretion > 0.3g. Exclusion criteria included diabetes; systemic lupus erythematosus; Henoch-Schonlein purpura; hepatic disease and the treatment with angiotensin-converting enzyme (ACE) inhibitors, angiotensin type-1 receptor (AT1R) blockers (ARBs), corticosteroids and immunosuppressive drugs. Fifteen healthy donors, without microscopic hematuria and proteinuria, were recruited as normal control subjects. 20 ml of whole blood was collected from each subject and serum was isolated via centrifuge and frozen at -20°C. Isolation, purification and characterization of IgA1 from sera with a jacalin-agarose affinity column were performed as previously described.¹⁵

Cell culture

Mouse mesangial cells and conditionally immortalized mouse podocytes were cultured.¹⁵ Briefly, mesangial cells were routinely maintained in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% fetal bovine serum and 0.6U/ml insulin. Passage 6-8 cells were used in the experiments. Podocytes were propagated in RPMI-1640 medium con-

taining 10% heat-inactivated FBS and 10U/ml mouse recombinant INF- γ (PeproTech, London, UK) under permissive conditions (33°C). Monolayers of podocytes at approximately 80% confluence were transfer to nonpermissive temperature (37°C) without INF- γ for differentiation. Podocytes at 37 °C were used in experiments after at least 12 days.

Adhesion assay

Podocytes were harvested with trypsin and suspended in mesangial cell-conditioned medium. In some wells, aIgA1 from IgAN patients or healthy donors was added to podocytes incubated with serum-free medium. An equal number of cells (4 $\times$ 10³/well) were seeded in rat-tail type I collagen (Sigma, St Louis, USA) pre-coated 96-well plates. In some wells, podocytes were pre-treated with valsartan (100 μ M) or neutralizing antibodies of TNF- α (50 ng/ml) for 1 hr. After treatment at 37 °C for 6 hr, two methods were used to evaluate the adhesive capacity of podocytes. All experiments were repeated three times to ensure reproducibility.

Cell counting

The number of the total cells in each well were quantified by counting manually. Then, cells were washed twice with ice-cold PBS and the number of adhered cells was counted. The percentage of adherent cells was calculated by the number of adhered cells divided by the total number of cells.

Hexosaminidase Assay: Non-adherent cells were removed by three washes with ice-cold PBS. Subsequently, 3.75 mM p-nitrophenol-N-acetyl--D-glucosaminide (Sigma, St Louis, USA) in 50 mmol/l citrated buffer (pH 5.0) with 0.25% Triton X-100 was added to each well for 1 hr at 37°C. And then, the enzyme was deactivated by adding 50 mM glycine and 5 mM EDTA (pH 10.4). Absorbance was read at 405 nm with a microplate reader.¹⁶

Real-time reverse transcription-polymerase chain reaction of ILK transcripts

Total cellular RNA was extracted from the podocytes using TRIzol Reagent according to the manufacturer's protocols. The RNA concentration and quantity were assessed by ultraviolet absorbency at 260 nm and agarose gel electrophoresis. Then, the procedure began with reverse transcription of total RNA to cDNA using oligo-(dT) primers, random primers and 400ng RNA. Subsequently, real-time RT-PCR was performed using Applied Biosystem 7000 real-time PCR System and SYBR® Premix Ex Taq™ Perfect Real time (TaKaRa). PCR conditions were as follows: 95°C (10s) denaturation together with 40 cycles of 95°C (5 s), 60°C (31 s). The PCR primer sequences of ILK were as follows: sense primer 5'-GGAAATACCTGG GACGGTAG-3' and anti-sense primer 5'-GACGCTC AGCAGACATGTGGA-3'. GAPDH was used as an internal quantitative control, which primer sequences were as follows: sense primer 5'- TGTGTCCGTCGT GGATCTGA -3' and anti-sense primer 5'-TTGCTGT TGAAGTCGCAGGAG-3'. Relative expression was calculated using delta-delta Ct ($\Delta\Delta Ct$) method with the formula $2^{-\Delta\Delta Ct}$ (where $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$).

Western Blotting

Lysates were prepared with cell lysis buffer (Cell Signaling Technology). The concentration of each protein sample was determined by a BCA method. Equal amounts of protein (30µg) were subjected to immunoblot analyses and separated in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane for blotting. After blocking for 1 hr at room temperature, PVDF membrane was incubated with rabbit anti-mouse ILK antibody (Santa Cruz, 1:400) overnight at 4°C. The secondary antibodies used were horse radish peroxidase-linked goat anti-rabbit IgG (H+L) antibodies (Cell Signaling

Technology) at 1:40,000 dilutions for 1 hr followed by ECL-mediated detection.

ELISA of Angiotensin II and TNF-α

Mesangial cells were incubated with or without aIgA1 for 48 hr, and then conditioned medium was collected. The medium was centrifuged at 1 000 rpm for 5 min to remove all the remaining cell pieces. Angiotensin II and TNF-α in the medium were detected by ELISA.

Statistical analyses

All data, unless otherwise stated, are expressed as mean ± standard deviation (mean ± SD). One-way factorial analysis ANOVA was conducted to determine the effect of conditioned medium between treated and control groups using SPSS 15.0. A two-tailed -level of 0.05 was used to determine statistical significance.

Results

Indirect effect of aIgA1 on the adhesive capacity of podocytes

First, we detected the adhesive capacity of podocytes using cell counting. The medium from mesangial cells incubated with aIgA1 from IgAN patients (PI group) reduced podocyte adhesion to collagen compared with medium from mesangial cells without aIgA1 incubated (CI group) ($35.0 \pm 4.8\%$ vs. $60.0 \pm 2.0\%$; $P < 0.05$). More podocytes became detached from collagen in the PI group (about 1.7-fold vs. CI group; $P < 0.05$). While the medium from mesangial cells without aIgA1 incubated (CI group) produced no effect on adhesiveness of podocytes compared with control medium (C group). (Fig. 1A)

However, 10µg/ml aIgA1 from IgAN patients or healthy control subjects did not exhibit any effect on adhesive capacity of podocytes (data not shown), thereby excluding a direct effect of possible residual aIgA1 on podocytes. Similar results were observed by using hexosaminidase assay (Fig. 1B).

FIGURE 1. Cell counting (A) and hexosaminidase assay (B) of podocytes incubated with IgA-mesangial cell conditioned medium. C, podocytes exposed to serum-free RPMI 1640 medium; CI, podocytes exposed to medium from mesangial cells incubated in serum-free RPMI 1640 medium without aIgA1. PI, podocytes exposed to medium from mesangial cells incubated with aIgA1 from IgAN patients; PI+V, podocytes pretreated for 1 hours with valsartan(10^{-5} M), and then exposed to medium from mesangial cells incubated with aIgA1 from IgAN patients; PI+T, podocytes pretreated for 1 hours with neutralizing antibodies of TNF- α (50 ng/ml), and then exposed to medium from mesangial cells incubated with aIgA1 from IgAN patients; NI, podocytes exposed to medium from mesangial cells incubated with aIgA1 from normal control subjects. * $P < 0.05$ vs. CI group, # $P < 0.05$ vs. PI group.

Indirect effect of aIgA1 on the expression of ILK

The relative mRNA level (normalized to GAPDH) of ILK in the PI group was 1.6-fold higher than in the CI group ($P < 0.05$). The medium from mesangial cells incubated with aIgA1 from both IgAN patients and normal donors up-regulated ILK synthesis in podocytes, while the former exhibited stronger effect when compared with each other ($P < 0.05$). (Fig. 2A)

FIGURE 2. mRNA (A) and protein (B) expression of ILK in podocytes incubated with IgA-mesangial cell conditioned medium. * $P < 0.05$ vs. CI group, # $P < 0.05$ vs. PI group. (Abbreviations see Fig 1 legend)

The protein level (normalized to GAPDH) of ILK in the PI group was 1.38-fold higher than in the CI group. To rule out the effect of possible residual aIgA1 on podocytes, aIgA1 from patients with IgAN or healthy control subjects was directly added into the serum-free medium for 12 hr. The result suggested that a low dose of aIgA1 did not have any effect on ILK synthesis (data not shown). (Fig. 2B)

Angiotensin II level in mesangial medium and its effect on podocytes

Angiotensin II increased in the specially treated medium from mesangial cells incubated with aIgA1 from IgAN patients (P group) compared with medium from mesangial cells incubated with aIgA1 from healthy control subjects (N group) and medium from mesan-

FIGURE 3. Expression of angiotensin II in medium from mesangial cells incubated with aIgA1. N, medium from mesangial cells incubated with aIgA1 (100 µg/ml) from normal control subjects. * $P < 0.05$ vs. C group or N group. (Abbreviations see Fig 1 legend)

FIGURE 4. Expression of TNF-α in medium from mesangial cells incubated with aIgA1. * $P < 0.05$ vs. C group or N group. (Abbreviations see Fig 1 and 3 legends)

gial cells incubated in serum-free medium without aIgA1 (C group) (1.56-fold increase vs. N group, 1.97-fold increase vs. C group, respectively. $P < 0.05$) (Fig. 3). Blockade of angiotensin II by valsartan can partially reverse the changes of ILK and adhesion of podocytes induced by the conditioned medium. (Fig. 1 and Fig. 2)

TNF-α level in mesangial medium and its effect on podocytes

The level of TNF-α increased in medium from mesangial cells incubated with aIgA1 from IgAN patients (P group) compared with medium from mesangial cells incubated with aIgA1 from healthy control subjects (N group) and medium from mesangial cells incubated in serum-free medium without aIgA1 (C group) (4.07-fold increase vs. N group, 2.85-fold increase vs. C group, respectively. $P < 0.05$) (Fig. 4).

However, using anti-TNF-α neutralizing antibody did not result in any change in integrin-linked kinase and adhesive capacity of podocytes (Fig. 1 and Fig. 2).

Discussion

In the present study, we showed that IgA-mesangial cell conditioned medium can inhibit adhesive capacity

and up-regulate ILK synthesis in podocytes. Moreover, the medium from mesangial cells incubated with aIgA1 from IgAN patients exhibited a more obvious effect compared with medium from mesangial cells incubated with aIgA1 from healthy control. We also examined the mediators (including angiotensin II and TNF-α) which are probably involved in the pathogenesis of podocytopenia. The process of communications between mesangial cells and podocytes was mediated by angiotensin II. Angiotensin II was increased in IgA-mesangial cell conditioned medium and inhibition of renin-angiotensin system by valsartan, a selective ARB, can partially reversed angiotensin II-induced podocytes adhesion defect to control levels. In the supernatants of mesangial cells incubated with or without aIgA1, TNF-α was increased in mesangial cells medium incubated with aIgA1 from IgAN patients. However, pre-treatment of podocytes with anti-TNF-α neutralizing antibody did not result in any protective effect on podocyte adhesion.

The hallmark of IgAN is the finding of mesangial deposition of pIgA1 complexes, an initial event in the development of this disease. Some studies have demonstrated that the podocyte may act as a key culprit in IgAN, and loss of podocytes directly connected to the severity of the disease.^{9,10,15}

The maintenance of podocyte injury and subsequent reduction of podocyte number would be crucial

in accelerating disease progression, eventually leading to glomerulosclerosis and glomerular destruction. The contribution of intramesangial deposition of pIgA1 to loss of podocytes is not clear. A recent study discovered a novel mechanism of glomerulo-podocytic communication in IgAN.¹⁷ This indicated that cytokines released from mesangial cells can have certain effects on podocytes, which implies a potential mechanism for initial deposition of pIgA1 in mesangial cells extends to podocytes.

Attachment of podocytes to the GBM, as a biological process, is an important prerequisite for survival and physiological functions of podocytes. Reduction of adhesiveness and subsequent loss of podocytes have been postulated to contribute to the development of glomerular sclerosis and loss of renal function.^{18, 19} A series of studies had showed that the majority of podocytes in urine sediments of patients with glomerular disease such as focal segmental glomerulosclerosis and lupus nephritis were viable.¹² These findings supported a critical role of detachment from the GBM in loss of podocytes, which may result in incompletely covering the external surface of the GBM and eventually leading to synechiae, the initially irreversible lesion of glomerulus.

The detailed mechanisms underlying reduction of podocyte adhesive capacity remain to be elucidated. Various molecules, including integrins and dystroglycans, have been implicated. Recent studies indicate that integrin-linked kinase may also be involved in podocytes adhesion.^{13,20,21} Increase of ILK level in glomeruli is crucially involved in the progression of several glomerular diseases such as diabetic nephropathy.²¹ ILK is considered a key regulator linking cytoplasmic domains of β -integrins and numerous cytoskeletal proteins.^{22,23} Thus, its dysregulation appears to affect the adhesion capacity of podocytes and eventually results in the detachment from GBM. In our experiment, ILK was up-regulated after 12 hr of conditioned medium co-incubation. Considering the key role of ILK in integrin-mediated cell-matrix interaction, our study suggests that up-regulation of ILK

may lead to a podocyte adhesion defect in IgA nephropathy. Further studies should be conducted to establish a direct link between ILK and the adhesive capacity of podocytes by using siRNA or other ILK inhibitory agents to inhibit the effect of ILK.

Based on our findings, we speculate that angiotensin II may be the key mediator in mesangial-podocyte communication. Angiotensin II is the final effector of the renin-angiotensin system (RAS). Blockade of RAS can reduce systemic and glomerular pressure, previously regarded as the major effect of ACE inhibitors and ARBs. Recent studies have demonstrated that locally synthesized angiotensin II in the kidney may be involved in tissue injury through a series of nonhemodynamic effects in many kidney diseases. Furthermore, both *in vitro* and *in vivo* studies have demonstrated a role for angiotensin II in promoting podocyte apoptosis. Chie Miyake-Ogawa et al.²⁴ revealed that the expression of RAS components, including ACE and AT1R, was up-expressed in kidney tissues of IgAN. Han SY et al.²⁵ reported that angiotensin II induced ILK overexpression and displayed inhibitory effects on podocyte adhesion *in vitro*. Our results show that valsartan, a commonly used ARB, had a benefit for podocyte adhesion, which suggests a potential role for angiotensin II in podocytopenia. This effect on podocytes illustrated a novel action of ARB for the use in IgAN.

In conclusion, our results demonstrate that IgA-mesangial cell conditioned medium incubated with aIgA1 from IgAN patients exhibited a higher inhibitory effect on podocyte adhesion and leads to up-regulation of ILK *in vitro* compared with the medium without aIgA1 incubated. Our study also suggests that blockade of angiotensin II can protect podocytes from detachment. These results indicate that IgA-mesangial cell conditioned medium promotes podocyte injury mainly via upregulation of angiotensin II. IgA1 from IgAN patients may inhibit adhesive capacity and up-regulate ILK synthesis in podocytes through indirect pathways.

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