Serum IgA1 from IgA nephropathy patients induces apoptosis in podocytes through direct and indirect pathways

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Abstract

Purpose: To investigate apoptosis of podocytes induced by IgA1 isolated from IgA nephropathy (IgAN) patients through direct and indirect pathways.

Methods: Jacalin affinity chromatography and Sephacryl S-200 molecular sieve chromatography were used to isolate IgA1 from blood of IgAN patients made as aggregated IgA1 (aIgA1). Podocytes were incubated with aIgA1 or special treated medium from mesangial cells after co-incubation with aIgA1 from IgAN patients. Apoptosis of podocytes was assessed by TUNEL staining and flow cytometry. Real-time PCR was used to detect the mRNA expression of Bcl-2, Bax, Fas and Fas-L.

Results: aIgA1 from IgAN patients induced more apoptosis of podocytes by both time and concentration-dependent patterns than control (30.5±5.4% vs 20.5±4.5, respectively, P<0.05). The percentage of apoptotic podocytes exposed to treated medium was higher than control (28.5±5.9 % vs 20.5±4.5%, respectively, P<0.05). The level of normalized Fas mRNA expression in podocytes exposed to aIgA1 was 2.4-fold higher than control (P<0.05), while the level in podocytes exposed with treated medium was 1.89-fold higher than control (P<0.05), and the level of normalized Bcl-2 mRNA expression in this group was 72% lower than control (P<0.05).

Conclusion: IgA1 from IgAN patients may induce apoptosis of podocytes through direct and indirect pathways. IgA1 may accelerate progression of IgAN by inducing apoptosis of podocytes.

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis, is one of the leading causes of renal failure in China.¹ IgAN runs a highly variable clinical course with a slow but progressive renal failure that occurs in 30% - 50% of patients within 30 yr of clinical presentation.², ³ The disease is characterized by mesangial deposition of polymeric IgA1 ⁴, proliferation of mesangial cells, increased synthesis of extracellular matrix, and infiltration of macrophages, monocytes and T cells.⁵ Although the pathogenesis of IgA nephropathy is not fully understood and its treatment is not well defined, existing evidence indicates that aberrantly glycosylated IgA1 is closely associated with pathologic phenotype of IgA.
nephropathy and is an important component in the pathogenesis of the disease.\textsuperscript{4,6}

The glomerular visceral epithelial cells, podocytes, are a group of highly specialized and terminally differentiated cells with limited mitotic capacity. Podocytes have limited ability to be replaced if lost. Reduced podocyte number seems to be a critical determinant underlying the development of glomerulosclerosis that leads to progressive renal failure in diabetic and non-diabetic renal disease. IgAN patients with urinary podocyte excretion show higher proteinuria and worse renal function. Urinary podocyte excretion in these patients is related to glomerulosclerosis and interstitial fibrosis. Podocyturia is not only clinical evidence of renal damage, but also the hallmark of IgAN activity.\textsuperscript{7} Lemley \textsuperscript{8} found that podocytopenia was associated with sclerosis of the glomerulus, decreased permselectivity and decreased glomerular filtration rate. In addition, when podocyte numbers decreased below approximately 250 cells per glomerulus, sclerosis became acute, indicating that podocyte loss is correlated with the severity of IgAN. The loss of podocytes is an important factor for progression of glomerulosclerosis and interstitial injury in IgAN patients.\textsuperscript{9} Clinical pathological correlations demonstrated that down-regulation of Bcl-2 expression in podocytes, the prototypic member of the Bcl-2 class of proto-oncogenes that blocks cell death without promoting cell proliferation, was associated with poor renal prognosis.\textsuperscript{10} Others have also shown that apoptosis is a major factor in the decrease of podocyte numbers.\textsuperscript{11} In our previous work, we found that IgA1 could inhibit the proliferation of podocytes and the expression of nephrin, which indicated that IgA1 might affect the biological events of podocytes. Can IgA1 induce apoptosis of podocyte? In this study, we found that IgA1 from IgAN patients induced apoptosis of podocyte through direct and indirect pathways.

Methods

Materials

Jacalin agarose was purchased from Sigma. Sephacryl S-200 was obtained from Amersham Biosciences. D- (+)-melibose was obtained from Fluck. RPMI 1640 medium was obtained from GIBCO BRL. Fetal bovine serum (FBS) was purchased from PAA. Mouse γ-interferon was obtained from PeproTech Company. Mouse anti-human IgA1 antibody was obtained from Southern Biotechnology. HRP-conjugated goat antimouse IgG antibody was purchased from Jackson Immuno Research. Annexin-FITC apoptosis detection Kit was obtained from BD Biosciences Pharmingen. In Situ Cell Death Detection Kit, POD was obtained from Roche. SYBR® Premix Ex Taq\textsuperscript{™} (Perfect Real time) was from TaKaRa.

Patients and control subjects: The study was carried out in accordance with principles of the declaration of Helsinki and was approved by the university and hospital ethics committees. Informed patient consent was obtained. Twenty-two Chinese patients 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 using immunofluorescence examination, and by the presence of mesangial electron-dense deposits by ultrastructural examination. All patients were symptomatic for more than 12 months and no serious renal impairment was documented. Systemic lupus erythematosus, Henoch-Schonlein purpura, and hepatic diseases were excluded by analyzing serum samples for the presence of anti-DNA antibodies, hypocomplementemia, or hepatitis B virus surface antigen, respectively, and by detailed clinical history and physical examination. 20 ml of blood was colleted from each patient at clinical quiescence. The serum was isolated and frozen at -20\textdegree C until isolation of IgA by jacalin-agarose affinity column.
Fifteen healthy volunteers, comparable in age and race, without microscopic hematuria and proteinuria, were recruited as normal control subjects. Serum was similarly collected from these individuals for processing. Informed written consent for blood sampling was obtained from every subject.

Isolation of Human IgA: IgA1 was isolated from pooled serum of IgAN patients and healthy donors by jacalin affinity chromatography. Briefly, Jacalin columns were prepared using Jacalin-immobilized agarose resin having an IgA1 binding capacity >2 mg/ml of gel. The pooled serum samples were diluted 1:1 with phosphate-buffered saline (PBS pH 7.4), filtered through a 0.2μm Corning syringe filter, then applied to the column, and washed with 175mM Tris-HCl (pH 7.4) until the optical density at 280nm was less than 0.01. Then IgA1 was eluted with 0.1M D-(+)-melibiose in 175 mM Tris-HCl (pH 7.4) until the optical density returned to 0.01. The eluted fractions were pooled, concentrated and applied to Sephacryl S-200 molecular sieve column. Three peaks appeared which represented three factions: polymeric IgA1 (pIgA1), monomeric IgA1 (mIgA1) and other non-IgA1 proteins. These fractions were pooled, concentrated and filtered with a 0.2 μm Corning syringe filter. Because the amount of pIgA1 recovered from the purification process was not sufficient for further analyses, we incubated the purified monomeric IgA1 at 63°C for 150 min to obtain aggregated IgA1 (aIgA1), as described previously. The transition from monomeric to aIgA1 was monitored using a Sephacryl S-200 column, and a single peak was observed after incubation at 63°C. The purity of IgA1 fractions was confirmed by SDS–PAGE analysis followed by Coomassie Blue staining. The samples were identified by Western blot and kept frozen at -70°C until used.

Cell culture: Two cell lines: mouse podocytes(MPC5) and mouse mesangial cells(MSC-1097) were provided by Professor Xueqing Yu. Experiments were performed using early-passage (10th to 18th), growth-restricted, conditionally immortalized MPC5 cell. The cells were grown in RPMI 1640 medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml). For passaging cells, podocytes were grown under "growth permissive" conditions, which involved growing cells at 33°C in the presence of IFN-γ (50 U/ml). To maintain undifferentiated cells, podocytes were grown under "restrictive conditions" in the absence of IFN-γ at 37°C with 95% air/5% CO2 for more than 12 days, followed by culturing in 6-well plates with or without glass cover slips at the same cell density. After arresting cell growth, the medium was removed and replaced with RPMI 1640 containing 0.5% FBS and varying concentrations of aIgA1. Starved podocytes were used in those studies described below.

Mouse mesangial cells (MSC-1097) were grown in RPMI 1640 medium containing 20% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (10 U/ml), at 37°C in 95% air/5% CO2. Starved MSC-1097 cells were cultured in 6-well plates with medium RPMI 1640 containing 0.5% FBS and aIgA1 (final concentration 100ug/ml) isolated from Patients with IgAN patients or healthy control for 48h. The cultured medium after co-cultured was collected to make as special treated medium for MPC5 and kept frozen at -70°C until used.

In all, the following experiments, cells were first cultured to 80% confluency, and then growth was arrested with culture medium RPMI 1640 containing 0.5% FBS for 18~24 h.

Experimental Design: We investigated the possible pathway of apoptosis in podocytes induced by aIgA1. First, we observed the direct pathway. Starved MPC5 cells were cultured in RPMI 1640 containing 0.5% FBS and aIgA1 (100 μg/ml) isolated from Patients with IgAN(aIgA1 group) or healthy control subjects(NaIgA group). Apoptosis rate of 100 μg/ml aIgA1 from patients with IgAN at different time
points(6h,12h,24h and 48h), different concentration of aIgA1(0, 50, 100,200, 400, 800,1600 μg/ml) for 24h from patients with IgAN were observed. We also observed the indirect pathway. Starved MPC5 cells were exposed to treated medium from starved MSC-1097 after co-culture with RPMI 1640 medium containing 0.5% FBS and aIgA1 (100 μg/ml) from patients with IgAN(PI Group) or healthy control subjects(NI group) for 24h. Apoptosis induced by 10% treated medium for 24h was observed. In our preliminary experiments, high concentrations of treated medium (>10%) severely damaged podocytes. Podocytes cultured in RPMI 1640 with 0.5% FBS were considered as controls (C group).

Assessment of Apoptosis

Flow cytometry: Flow cytometry was used to assess apoptosis of podocytes (Annexin-FITC apoptosis detection Kit, BD Biosciences Pharmingen). First, cells were washed twice with cold PBS, and then were scraped into 1×binding buffer to make cell suspension at a concentration of 1×10⁶ cells/ml. 100μL of cell suspension were transferred to a 5ml tube. With 5μL of Annexin V-FITC and 5μL of Propidium Iodide, the cells were gently mixed and incubated for 15 min at room temperature in the dark. Then, to each tube were added 400μL of 1×binding buffer, and analyzed by flow cytometry (BD FACSCalibur) within 1h, with excitation at 488 nm and emission collected at 525 nm (FITC-conjugated AV-labeled cells) and 620 nm (PI-labeled cells). Single labeling was used to gate and control for bleed-through. The cell population was characterized according to whether it was labeled with neither AV nor PI (viable), PI alone (necrotic), AV alone (early apoptotic), or both PI and AV (late apoptotic) The apoptosis rate of MPC5 cells were summarized as early apoptotic plus late apoptotic.16 are from flow cytometry analysis if there is no special indication.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) technique (In Situ Cell Death Detection Kit, POD, Roche ) was also used to validate the apoptosis of MPC5 cells. Briefly, air dried cell samples were fixed with a freshly prepared 4% paraformaldehyde in PBS(pH=7.4) for 1h at 15-25°C, then incubated with 3% H2O2 for 10 min at 15-25°C and in 0.1 Trixon in 0.1% sodium citrate for 2 min on ice, then 50μl TUNEL reaction mixtures were added to each sample(positive control: fixed and permeated cells were incubated with 30u/ml DNase I for 10 min at 15-25°C; negative control. Fixed and permeated cells were incubated in 50ul/well label solution(without terminal transferase) instead of TUNEL reaction mixture), After adding the lid, cells were incubated for 60 min at 37-°C in a humidified atmosphere in the dark. Analysis was performed with fluorescence microscope (Nikon), and apoptosis rate of MPC5 cells was indicated as (the number of nucleus under microscope/the number of nucleus of positive control) ×100%.

Total RNA isolation and reverse transcription: Total RNA was extracted from cells using Trizol (Invitrogen) according to the protocol provided by the manufacturer. The extracted RNA was suspended in ribonuclease-free water and was quantified by measuring the absorbance at 260 nm. To ensure samples were free of degradation, the 28S and 18S rRNAs were examined by ethidium bromide staining following agarose gel electrophoresis. Total RNA (500ng) of each group was reverse transcribed using the reverse transcriptase (RT) provided in the SYBR® Premix Ex Taq™ (Perfect Real time)(TaKaRa), the conditions of reaction were as following: 37°C for 15min, then 85°C for 5 sec.

Real-time PCR: The primers used in the experiments are summarized in the following {Sequence (5’→3’)}: Bcl-2(Sense: GTGTTCATGCACCAAGTCCA; Anti-sense: AGGTACAGCATTGCGCATATA , length: 127bp); Bax (Sense: CAGATGCGTCCACGATAACT; Anti-sense: GTTGAAGTGCATACAACACAC, length: 165bp); Fas(Sense: GAA
Using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and SYBR® Premix Ex Taq™ Perfect Real time (TaKaRa), PCR was performed in 20 μl total volume for each sample, containing 10 μl SYBR® Premix Ex Taq™, 0.4 μl primer, 0.4 μl ROX Reference Dye, 2 μl cDNA and 6.8 μl dH2O. The amount each primer pair added to the PCR reactions was determined by preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions were as follows: initial denaturation at 95-°C for 10 sec, followed by 40 cycles of denaturation at 95-°C for 5 sec and annealing/extension at 60-°C for 31 sec. Each sample was analyzed in triplicate and the gene products were quantified and normalized to the GAPDH gene products. After real-time PCR, the temperature was increased from 60-°C to 95-°C at a rate of 2°C/min to obtain a contrasting melting curve. A control lacking input cDNA content of each sample was determined using the comparative C_T method with 2^-ΔΔCT. The results were presented as relative expression normalized to the GAPDH gene and expressed in arbitrary units. Signals from the control group were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels to reveal a single band.

**Statistical analysis:** All data are expressed as mean ± standard deviation unless otherwise specified. Statistical differences were assessed using multivariate analysis of variance for repeated measures. All P values quoted are two-tailed, and significance was defined as P<0.05.

**Results**

1. **The effect of alglA1 (100μg/ml) on podocyte apoptosis for 24h.**

The percentage of apoptotic podocytes from cultures exposed for 24 h to alglA1 from IgAN was higher than the percentage of apoptotic podocytes grown for 24 h in control medium (30.5±5.4% vs 20.5±4.5, respectively, P<0.05). Whereas, the percentage of apoptotic podocytes from cultures exposed for 24 h to alglA1 from IgAN was slightly, but not not significantly,
lower than that from healthy controls (30.5±5.4% vs 31.4±5.3, respectively). Similar results were also found in TUNEL staining. (Fig-1 and Fig-2).

2. The effect of aIgA1 (100μg/ml) on podocyte apoptosis at different times.

100μg/ml aIgA1 from IgAN induced MPC5 cells apoptosis in a time-dependent pattern. The percentage of the cell apoptosis at 12 hr was higher than at 6 hr ($P<0.05$), and at 24 hr was higher than at 12 hr ($P<0.05$), and at 48 hr was higher than at 24 hr ($P<0.05$) (Fig-3).

3. The effect of different concentration of aIgA1 on podocyte apoptosis for 24h.

aIgA1 from IgAN induced MPC5 to apoptosis in a concentration-dependent pattern. The percentage of apoptosis of podocytes induced by 50μg/ml of aIgA1 was not higher than control. However, the rate in podocytes, at more than 100μg/ml of aIgA1, was higher than control. (Fig-4)

4. The effect of mesangial culture medium on podocyte apoptosis for 24h (indirect pathway).

The percentage of apoptotic podocytes from cultures exposed for 24 hr to medium from mesangial cells cultured with aIgA1 isolated from IgAN patients was higher than in the control group (28.5±5.9 vs20.5±4.5, $P<0.05$); whereas the percentage of apoptotic podocytes from cultures exposed for 24 h to medium from mesangial cells cultured with aIgA1 isolated from normal was not significantly higher than from those grown for 24 h in control medium (22.5±5.8 vs20.5±4.5). Similar results were found in TUNEL staining. (Fig-1 and Fig-2).

5. The effect of mesangial culture medium on podocyte expression of Bcl-2, Bax, Fas and Fas-L mRNAs.

First, we detected changes in apoptosis related genes of podocytes. The relative levels of normalized Bcl-2 mRNA in podocytes of PI group was 72% lower than normal ($P<0.05$). Levels of the other three groups was not significantly different from control. The level of normalized Bax mRNA expression was not different in these four groups compared with control. The level of normalized Fas mRNA expression in podocytes of PIgA and NIgA group was 2.4-fold higher than control ($P<0.05$), and the level in podocytes of PI group was 1.89-fold higher than control ($P<0.05$). Meanwhile, the levels of podocytes in NI group was not different from control. The level of normalized Fas-L mRNA expression in podocytes of PIgA and NIgA group was 80% lower than control ($P<0.05$), and the
level of podocyte of PI group was 75% lower than control ($P<0.05$), while the level of podocyte of NI group was not different from (Fig-5).

**Discussion**

We found that IgA1 from IgAN patients can induce apoptosis of podocytes in a time and concentration-dependent pattern indicating that IgA might accelerate the progression of IgAN by inducing apoptosis of podocytes. This had little chance of occurring at the commencement of the disease since glomerular basement membrane (GBM) blocked larger molecules of pIgA1. However, pIgA1 can leak from GBM and contact with podocytes when GBM was seriously damaged. At that time, IgA1 could induce apoptosis of podocytes and accelerate progression of IgAN. We also found that the medium from mesangial cells incubated with algA1 from IgAN patients can induce apoptosis of podocytes compared with other medium and control. This was more likely to occur during the pathogenesis of IgAN. The results indicated that IgA1 can induce apoptosis of podocytes through direct and indirect pathways which has not been reported previously.

IgAN was characterized by IgA deposition in mesangium, which can stimulate the production of many cytokines such as IL-6, TNF-$\alpha$, TGF-$\beta$1 and angiotensin-II (Ang-II). These cytokines are excreted into the medium of mesangial cells (isolated from

![FIGURE 5. Real-time PCR analysis of mRNA expression of apoptosis-related genes in podocytes (*$P<0.05$): A: Bcl-2/GAPDH mRNA; B: Bax/GAPDH mRNA; C: Fas/GAPDH mRNA; D: Fas-L/GAPDH mRNA.](image-url)
IgAN patients) when the cells were cultured in the presence of aIgA1. Previous studies have shown that TGF-β1 and Ang-II can induce apoptosis of podocytes. Thus, we examined whether medium from mesangial cells co-cultured with aIgA1 from IgAN induced apoptosis of podocytes. This is the first study to determine that the medium from mesangial cells cultured with aIgA1 from IgAN patients confers apoptosis of podocytes, whereas the medium from mesangial cells cultured with aIgA1 from healthy cells does not. This suggests that IgA1 accelerated disease progression of IgAN by inducing apoptosis of podocytes through mesangial cell pathway, and implicates communication between podocytes and mesangial cells. Further study is required to investigate which molecules are involved in the communication between podocytes and mesangial cells.

IgA1 plays an important role in the pathogenesis of IgA nephropathy. Previous studies focused on mesangial cells incubated with aIgA1 from IgAN patients. However, podocyte damage was also found in IgAN and played a role in the progression of the disease. Recent studies have pointed out that a reduction in the podocyte number directly causes proteinuria and glomerulosclerosis. Maccoon studied the relationship between glomerular podocyte numbers and the development of proteinuria and glomerulosclerosis in male Munich Wistar Fromter (MWF) rats. Their findings suggest that the loss of podocytes is directly related to podocyte dysfunction and to the reduction in glomerular permeselectivity that ultimately leads to extensive proteinuria and renal scarring.

Emerging experimental and clinical studies show that apoptosis was a major cause of reduced podocytes number, which leads to proteinuria and glomerulosclerosis. Bottinger was the first to show that apoptosis of podocytes increased in experiments performed in tumour growth factor transgenic mouse. Wiggins and co-workers showed that podocyte apoptosis occurs in puromycin aminonucleoside–induced nephrosis. In addition, they found that a 20% reduction in the number of podocytes is required to detect glomerulosclerosis, and that any further reduction is directly correlated with increased sclerosis. Also, they found that podocyte damage induced further podocyte damage in a detrimental and autonomous cycle that led to localized spreading of glomerulosclerosis. It is commonly accepted that in many glomerular diseases, podocyte damage, either originating from within or from an external source, initiates a sequence of events that results in sclerosis.

In general, caspases are the final effectors of apoptosis and the apoptotic pathways that are typically classified according to which pro-caspase is activated. For example, activation of pro-caspase 8 occurs via death-receptor signaling. The integral membrane protein Fas is one such death receptor and it transmits apoptotic stimuli to downstream molecules such as FADD. Fas-FADD-caspase 8 pathway has been implicated in renal tubular cell apoptosis during endotoxemia following ischemic injury, transplant rejection and in the tubular atrophy of chronic renal failure. However, activation of the initiator pro-caspase 9 is dependent on mitochondrial signaling pathways involving members of the Bcl-2 family such as Bax and Bak. Both pathways may be involved in apoptosis of epithelial cells. Therefore, we chose to examine the following apoptosis-related genes from these two apoptotic pathways: Fas and Fas-L, Bcl-2 and Bax. We found that Fas mRNA of podocytes was up-regulated when these cells were exposed to aIgA1 directly, while the Fas-L mRNA was slightly down-regulated indicating that death receptors such as Fas may be involved in apoptosis of podocytes by aIgA1 isolated from IgAN patients and normal control. While the pathway of podocyte apoptosis induced by medium from mesangial cells cultured with aIgA1 was complex, down-regulation of Bcl-2 and up-regulation of Fas may be found in podocytes exposed to medium from mesangial cells cultured with aIgA1 isolated from IgAN patients. This indicates that death receptor and mitochondrial signaling pathways might be involved in apoptosis of podocytes by medium of

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mesangial cells. The complexity of apoptotic pathway in podocytes induced by medium from mesangial cells cultured with αIgA1 isolated from IgAN patients might be due to the presence of various apoptosis-inducing factors in the medium, such as TGF-β1 and Ang-1. Further study is required to explore the pathways of podocyte by different IgA1.

In conclusion, we report that IgA1 induces apoptosis of podocytes by direct and indirect pathways, and that IgA1 may accelerate progression of IgAN by inducing apoptosis of podocytes.

References


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