Proliferation and Cytokine Production of Human Mesangial Cells Stimulated by Secretory IgA Isolated from Patients with IgA Nephropathy

Yan Liang, Junjun Zhang, Yali Zhou, Guolan Xing, Guoqiang Zhao, Zhangsuo Liu

Department of Nephrology, First Affiliated Hospital of Zhengzhou University, Research Institute of Nephrology, Zhengzhou University, Key-Disciplines Laboratory Clinical-Medicine Henan, College of Basic Medical Sciences, Zhengzhou University, Zhengzhou, China

Key Words
IgA nephropathy • Secretory IgA • Saliva • Mesangial cells • Cytokines • miR-16

Abstract
Background/Aims: IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis, and often aggravates by mucosal infection. Secretory IgA (SIgA) is the dominant immunoglobulin in mucosal immunity, and is deposited in the mesangium in IgAN. The biological effects of SIgA on mesangial cells are poorly understood. Methods: Deposition of SIgA in frozen renal sections from IgAN patients was detected and the association between deposition of SIgA and patients characteristics was analyzed. The biological effects of SIgA and polymeric IgA (pIgA) on human renal mesangial cells were compared. We also studied the molecular mechanism of microRNA regulating the inflammatory effects of SIgA on mesangial cells. Results: Fifty-five of 176 patients had SIgA deposition with higher incidence of infection history and hematuria, lower serum cystatin C, β2 microglobulin, blood urea nitrogen and T-grade in the Oxford classification, compared with patients without SIgA deposition. SIgA stimulated mesangial cells at a higher ratio of proliferation and higher production of interleukin (IL)-6, IL-8, monocyte chemotactic protein 1, transforming growth factor-β1 and fibronectin, compared with SIgA from healthy volunteers. The proliferation and cytokines production in mesangial cells stimulated by SIgA were significantly lower than that stimulated by pIgA. miR-16 targeted the 3’-untranslated region of IL-6 and suppressed its translation in mesangial cells induced by SIgA. Conclusions: The biological effects of SIgA on mesangial cells differ from those of pIgA. SIgA stimulates mesangial cell proliferation and production of proinflammatory cytokines. IL-6 production is regulated by miR-16 in mesangial cells.

Y. Liang and J. Zhang contributed equally to this paper.

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Introduction

IgA nephropathy (IgAN) is the most common of primary glomerulonephritis, characterized by the predominant deposition of IgA in the renal mesangium [1, 2]. The clinical association between exacerbation of IgAN and mucosal infection has supported the view that IgAN might be connected with mucosal immune responses. Secretory IgA (SIgA) is the dominant immunoglobulin in mucosal immunity and represents the major humoral defense mechanism of mucosal areas including the oral cavity, respiratory system, intestinal cavity and colostrum [3, 4]. Small amounts of SIgA are found in normal serum and levels are elevated in some disorders including liver disease and human immunodeficiency virus infection [5]. Our previous study showed SIgA in serum was associated with creatinine clearance, proteinuria and renal pathological phenotypes. In addition, it was deposited in ~33% of patient kidneys, suggesting SIgA might play an important role in the pathogenesis of IgAN [6].

Currently, the pathogenesis of IgAN remains unclear. Previous studies focused on the pathogenic role of aberrant glycosylated IgA in IgAN [7-9], which is deposited in mesangial areas to induce various immune inflammatory responses in IgAN, including activation of the complement pathway and stimulation of mesangial cells to produce cytokines and chemokines [10-13]. However, whether patients with SIgA deposition have similar clinical pathological characteristics to patients with IgA deposition, and whether SIgA has a similar pathogenic role to IgA in IgAN are unknown. Recently, some studies began to explore the abnormal expression of gene in IgAN [14, 15]. microRNAs (miRNAs) modulate protein expression by targeting mRNA transcripts and triggering translation repression or RNA degradation [16]. miRNAs regulate many biological processes including cell proliferation, inflammatory responses and apoptosis, and recent insights reveal that they might have important roles in the pathogenesis of IgAN [17]. To investigate further the biological effects of SIgA on human mesangial cells and its mechanism of action, we compared the characteristics of patients with and without renal SIgA deposition. We observed the production of cytokines and cell proliferation in human mesangial cells stimulated by SIgA and polymeric IgA (pIgA) isolated from IgAN patients. Finally, we studied the molecular mechanism of miRNA regulation of the inflammatory effects of SIgA on mesangial cells.

Materials and Methods

Patients

This study enrolled renal biopsy-proven IgAN patients in our department from January 2011 to June 2012. They were collected based on significant deposition of IgA but absence of IgM in glomerular mesangium by routine direct immunofluorescence, and the number of glomeruli in renal biopsy was >10 per patient. Patients with diabetes mellitus, active hepatitis, liver cirrhosis, severe metabolic syndrome, Henoch-Schönlein purpura, systemic lupus erythematosus and secondary IgAN-related diseases were excluded. The clinical parameters of all patients were obtained before renal biopsy. The estimated glomerular filtration rate (eGFR) was calculated using the four-variable Modification of Diet in Renal Disease (MDRD) formula if the age of patient was ≥18 years and the modified Counahan-Barratt formula if <18 years [18, 19].

Ethics Statement

Patients provided written informed consent and the study was approved by the Institutional Review Board of the First Affiliated Hospital, Zhengzhou University, China. All investigations were conducted according to the last version of the Declaration of Helsinki.

Routine renal histopathology

Staining for IgG, IgA, IgM and C3 on fresh frozen renal tissue was using direct immunofluorescence. For light microscopy, paraffin sections were stained with hematoxylin & eosin, periodic acid-Schiff, methenamine silver and Masson trichrome. Sections were evaluated by two experienced pathologists blinded to clinical data.
The severity of renal histological lesions was evaluated according to the Oxford classification, that is mesangial proliferation (M), segmental glomerulosclerosis (S), endocapillary hypercellularity (E) and tubular atrophy/interstitial fibrosis (T) [20].

**Detection of renal deposition of SIgA by immunofluorescence**

Unfixed renal tissues were embedded in optical cutting temperature compound (Sakura Tissue-tek, Bayer, Torrance, CA, USA). Subsequently, sections cut at a thickness of 4-µm were fixed in cold acetone. Sections were incubated in phosphate buffered saline (PBS) with 1% bovine serum albumin for 30 min. Mouse anti-human secretory component (SC, monoclonal, Genetex, San Antonio, CA, USA; dilution 1:100) was incubated overnight at 4°C as the primary antibody. Washed in PBS, and Alexa Fluor 594-labeled donkey anti-mouse IgG (Invitrogen, Paisley, UK) diluted at 1:100 was used as secondary antibody for 60 min at 37°C. Re-washed in PBS, fluorescein isothiocyanate-labeled rabbit anti-human IgA (polyclonal, Dako, Glostrup, Denmark; dilution 1:30) was added to the sections for 60 min at 37°C. All sections were viewed using a confocal microscope (LSM 710; Zeiss, Oberkochen, Germany). Negative controls were performed by omitting or replacing the primary antibodies with PBS.

**Collections of saliva and serum**

After the detection of immunofluorescence, we asked patients with SIgA deposition to leave a saliva sample. Before collection, each patient rinsed their mouth with sterile water. Between 6 am and 8 am, a sterile cotton roll was chewed for 2 min to stimulate saliva flow for each fasted patient and then saliva was collected into a sterile plastic tube. Samples were transported on ice to the laboratory and centrifuged at 4000 ×g for 15 min at 4°C. The supernatants were used to purified SIgA and stored at −80°C until processing. We collected and stored saliva from healthy volunteers who were age and gender matched, without hepatitis or kidney disease and without recent mucosal infection history, as controls.

On the morning of the renal biopsy (for IgAN patients who matched the before mentioned criteria) or healthy volunteers, 10 ml blood was collected from each enrolled subject. Plasma was isolated and frozen at -80°C immediately.

All the patients and healthy individuals signed the written consent forms before specimen collecting.

**Isolation of SIgA from saliva**

SIgA was purified from pooled saliva samples of patients (P-SIgA) and pooled saliva of healthy volunteers (N-SIgA) using jacalin affinity chromatography. The samples were diluted 1:1 with PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4), filtered through a filter membrane (0.22 µm pore diameter; Millipore, Bedford, MA, USA) and applied to a jacalin column prepared using commercially available jacalin immobilized on crosslinked 6% beaded agarose (Thermo Scientific, Rockford, IL, USA) with an IgA binding capacity of 1-3 mg/ml of gel. The column was washed with PBS until the optical density at 280 nm was < 0.1. SIgA was eluted with 0.1 M melibiose (Sigma, St. Louis, MO, USA) in PBS in 2.0 ml fractions until the optical density returned to 0.1. The concentrated sample was dialyzed against 0.01 M PBS (pH 7.4) for 24 h to remove melibiose.

**Identification of purified SIgA**

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE): commercial SIgA (AbD Serotec, Oxford, UK) and purified SIgA from IgAN patients and healthy volunteers in sample loading buffer were electrophoresed by 12% SDS-PAGE at 20 mA under reducing conditions. Consequentially, the gel was used for Mem Gel Staining (Applygen Company, Beijing, China) according to the instruction manual, similar to Coomassie Blue Fast Staining.

Western blot: SDS-PAGE procedures were the same as above and the gel was used for western blot. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking for 1 h at room temperature in blocking buffer (0.01 M Tris-HCl, pH 7.2, 0.15 M NaCl, 0.1% Tween-20, 20 g/l skimmed milk), the membrane was divided into two parts and incubated with monoclonal mouse anti-human SC (monoclonal, Sigma, dilution 1.4 μg/ml), IgA1 (monoclonal; immunogen: IgA1 heavy chains, formats available: purified IgG conjugated to horseradish peroxidase, AbD Serotec; dilution 1:2000), at 4°C overnight, respectively. After washed in Tris-buffered saline with Tween-20 (TBST), one part of the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Zhongshan
Biotech, Beijing, China; dilution 1:5000), for 1 h at room temperature. Re-washed, the two pieces of membrane were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). Western blotting images were scanned on a flatbed scanner using the ImageQuant scanner (LAS 4000 mini; GE Healthcare, Chalfont St Giles, UK).

**Isolation of polymeric IgA**

In order to compare biological effects of IgA and SIgA on mesangial cell, plgA were purified from pooled blood of IgAN patients (P-plgA) and pooled blood of healthy controls (N-plgA). IgA was first purified from plasma by a jacalin column, as isolated SIgA from saliva. Purified IgA was applied to a Sephacryl S-300 gel filtration chromatography column (GE Healthcare Life Sciences, Uppsala, Sweden) as previously described [21]. At 280 nm, plgA was high molecular mass IgA with molecular weights between 250 and 1000 kDa. Monomeric IgA was low-molecular mass IgA with molecular weights between 100 and 250 kDa [22]. plgA was pooled separately and concentrated. The purified P-plgA and N-plgA were identified by western blotting.

**Cell culture**

Primary human renal mesangial cells (HRMCs) were purchased from ScienCell™ Corporation (Carlsbad, CA, USA). Cells were cultured according to the manufacturer’s specifications in mesangial cell medium (MCM) supplemented with mesangial cell growth supplement, 5% fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 U/ml) (all purchased from ScienCell™) at 37°C in a humidified 5% CO₂ incubator.

**Detecting proliferation of mesangial cells stimulated by SIgA or plgA**

HRMCs were trypsinized and seeded in 96-well tissue culture plates (Costar, Corning, NY, USA) at a density of 1 × 10⁵ cells/ml in 100 μl MCM containing medium for 24 h. Cells were serum starved in MCM but without FBS for 24 h. The cells were washed and stimulated using P-SIgA, N-SIgA, P-plgA and N-plgA respectively. Controls were incubated with medium only (MCM with no FBS). Three replicate wells were used for the control and test concentrations. At first, the concentrations of SIgA and plgA were 50, 100, 200 or 400 μg/ml. We identified a suitable concentration (200 μg/ml), which was used to stimulate mesangial cells. The cells were cultured in an incubator (5% CO₂ at 37°C) for 12, 24 or 36 hours. Cell proliferation assay was assessed using the cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) in a microplate reader. To prevent the influence of purified SIgA and plgA, supernatants were dropped. Ten microliters of CCK-8 and one hundred microliters MCM without FBS were added to each well immediately, and the microplates were incubated at 37°C for 2 h in a 5% CO₂ humidified incubator. The absorbance was then measured at 450 nm using a microplate reader (Mark, Bio-Rad, Hercules, CA, USA). The experiments were repeated 3 times. The cell proliferation assay was calculated as follows:

\[ \text{Cell proliferation ratio} = \frac{OD(\text{test}) - OD(\text{blank})}{OD(\text{control}) - OD(\text{blank})} \times 100 \]

**Cytokines detection by enzyme-linked immunosorbent assay**

For measurement the protein release of cytokines, HRMCs were cultured in 6-well tissue culture plates (1 × 10⁶ cells/well) (Costar, Corning, NY, USA). The methods of incubation and stimulation were the same as for the proliferation assay. At 12, 24 and 36 h, culture supernatants were collected and stored at −80°C. The experiments were repeated 3 times. We used sandwich enzyme-linked immunosorbent assay (ELISA) kits for measurement of interleukin (IL) -6 (eBioscience, Vienna, Austria), IL-8 (R&D Systems, Minneapolis, MN, USA), monocyte chemotactic protein-1 (MCP-1; R&D Systems), transforming growth factor-β1 (TGF-β1; R&D Systems), tumor necrosis factor-α (TNF-α; R&D Systems) and fibronectin (eBioscience), according to the manufacturer’s specifications.

**mRNA expression of cytokines detection by real-time reverse transcriptase-PCR**

At 24 h incubation with SIgA or plgA, total RNA was extracted from cultured cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed to cDNA using the PrimerScript RT reagent kit (Takara, Shiga, Japan). The cDNA was stored at −20°C until further amplification. Gene
transcription levels of IL-6, IL-8, MCP-1, TGF-β1, TNF-α, fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined by real-time PCR using SYBR Green I Master (LightCycler® 480; Roche, Basel, Switzerland) on a Real-time PCR Instrument (LightCycler® 480 II; Roche). The relative mRNA expression of cytokines was calculated using the 2ΔΔCt method with the following formula: cytokine mRNA = 2−ΔΔCt, in which ΔΔCt = [(Ct cytokine - Ct GAPDH) experimental sample - (Ct cytokine - Ct GAPDH) control sample]. The primer sequences used in this study are shown in Table 1.

Expressions of miR-16 detected by quantitative real-time PCR
To detect the expressions of miR-16 in HRMCs stimulated by P-SIgA and N-SIgA, quantitative real-time PCR (qRT-PCR) was performed using a High-Specificity qRT-PCR Detection Kit (Stratagene, La Jolla, CA, USA) in conjunction with an ABI 7500 thermal cycler according to the manufacturer’s recommendations. qRT-PCR primer sequences are shown in Table 1. We used U6 small nuclear RNA (U6 snRNA) as an endogenous control for normalization. The relative expression of miR-16 was calculated using the 2−ΔCt method: in which ΔCt = (Ct miR-16 - Ct U6).

Prediction of miRNA targets
miR-16 is predicted to be a critical regulator in inflammatory responses of mucosal immunity [23]. In this study we used TargetScan and miRanda to predict miR-16 targets, which were related to proinflammatory cytokines.

Table 1. Primer sequences for real-time RT-PCR. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IL: interleukin; MCP-1, monocyte chemotactic protein-1; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α

| Gene     | Primer                                      | Accession number |
|----------|---------------------------------------------|------------------|
| GAPDH    | Forward 5'-TGTTGCCATCAATGACCCTT3'           | NM_002046        |
|          | Reverse 5'-CTCCACGAGCTACTCAGCG3'            | NM_001256799     |
| IL-6     | Forward 5'-GTTGTTAATGGGCATTCTT3'            | NM_000600        |
|          | Reverse 5'-ATAGTGTCTCAAGGCTCATAC3'          |                  |
| IL-8     | Forward 5'-TGAGAGGGGCTGAGAAATCTA3'          | NM_000584        |
|          | Reverse 5'-GCAACCTACAACAGACC3'              |                  |
| MCP-1    | Forward 5'-GACTAACACGAAACATCAA3'            | NM_002982        |
|          | Reverse 5'-GAATGAGGTTGCTGCTAT3'             |                  |
| TGF-β1   | Forward 5'-ATCATTTCAGTCACCATAGCA3'          | NM_000660        |
|          | Reverse 5'-GCAGGAACCTCCTTCTTA3'             |                  |
| TNF-α    | Forward 5'-ACCTGGGATTACAGGAATG3'            | NM_000594        |
|          | Reverse 5'-AGATGTCAGGGATCAAACG3'            |                  |
| Fibronectin | Forward 5'-TTCAAGAGACTGGGACGTCTT3'        | NM_054034        |
|          | Reverse 5'-CCACTTGAGCTGGATAGG3'             |                  |
| hsa-miR-16 | RT-primer 5'-TCGTATCCAGTGCGGCGTCCGTATT    | MIMAT004518      |
|          |                                             | CGACTGGATACGGGTTATA 3'     |
|          | Forward 5'-TCCGAATTGCTGTGTCAT3'             |                  |
|          | Reverse 5'-GTGCGAGGGTGGCCAGGT3'             |                  |
Dual-luciferase assay

The human IL-6 fragments containing putative binding sites for miR-16 were amplified by PCR from human genomic DNA (forward 5′-CAA CTC GAG CAT GGG CAC CTC AGA TTG TTG TTG T-3′, reverse 5′-ACC TCT AGA CCA CTT TCC ATT ATT ATT TCA AAA C′-3′). The mutant IL-6 3′-untranslated regions (UTRs) constructed with the TAATATT to CTTCCAA mutation were obtained by overlap extension PCR. The fragments (256 bp) were cloned into a pmirGLO reporter vector (Promega, Madison, WI, USA) with XhoI and XbaI, downstream of the luciferase gene, to generate the recombinant vectors pmirGLO-IL-6-wt and pmirGLO-IL-6-mut. For the luciferase reporter assay, HRMCs were transiently co-transfected with miRNA (miR-16 mimic or scrambled miR-16 negative control) and reporter vectors (wild-type reporter vectors or mutant-type reporter vectors), using Lipofectamine 2000. Luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to manufacturer’s instructions at 48-h post-transfection.

Endogenous regulation of miR-16 on IL-6 in HRMCs

HRMCs were divided into three groups: the nontransfected blank group (blank), scramble and mimic. The latter two groups were transfected with scrambled miR-16 and miR-16 mimic, respectively. After 48 h, three groups of cells were induced by P-SIgA purified at the previous step (200 μg/ml). 24 h later, total RNA was collected to detect IL-6 mRNA (as previously described) and the supernatants were collected to measure IL-6 protein. The protein levels of IL-6 were detected by western blot as described before. The first antibody was IL-6 (polyclonal; Abcam, USA; dilution 1:500) and the second antibody was HRP-conjugated goat anti-rabbit IgG (Zhongshan Biotech, Beijing, China; dilution 1:2000). An antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as an endogenous reference.

Statistical analyses

The normally distributed data were expressed as mean ± standard deviation (SD); skewed data were expressed as median with range, and categorical data were expressed as frequencies and percentages. For continuous variables, independent-sample t-test or one-way analysis of variance was used if the data were in normal distribution, and if not, a Mann–Whitney U-test was performed. Differences for categorical data were performed by chi-square Test. A P-value of <0.05 was considered significant. Analysis was performed with SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

Results

Glomerular deposition of SlgA in patients with IgAN

There were 176 patients enrolled in this study. Mesangium IgA deposition was detected in all patients. Significant mesangium SC deposits were detected in 55 patients (31.25%). The renal deposition of IgA and SC could be largely merged in these patients as observed by confocal microscopy (Fig. 1). The patients with SlgA deposition were called the SlgA-positive group, those without SlgA deposition were identified as the SlgA-negative group.

Fig. 1. Colocalization of secretory component and IgA detected by immunofluorescence. These were detected in frozen sections of a patient with IgA nephropathy (magnification × 200). Bar = 50 μm. A. Mesangial deposition of IgA (green). B. Deposition of secretory component (red). C. Merging of IgA and secretory component (yellow).
Table 2. Clinical and pathological characteristics of the SIgA-positive and SIgA-negative groups. SIgA-positive group: patients with SIgA deposition in renal; SIgA-negative group: patients without SIgA deposition in renal; SBP: systolic blood pressure; DBP: diastolic blood pressure; ALB: serum albumin; BUN: blood urea nitrogen; SCr: serum creatinine; eGFR: estimate glomerular filtration rate; β2–MG: β2 microglobulin. The severity of renal histological lesions was evaluated according to the Oxford classification: mesangial proliferation (M), segmental glomerulosclerosis (S), endocapillary hypercellularity (E) and tubular atrophy/interstitial fibrosis (T). The normally distributed data were expressed as mean ± SD; skewed data were expressed as median and range. *P < 0.05

|                          | SIgA-positive group (n = 55) | SIgA-negative group (n = 121) | P-value |
|--------------------------|------------------------------|-------------------------------|---------|
| Sex (male/female)        | 35/20                        | 81/40                         | 0.668   |
| Age (years)              | 31.73±12.96                  | 32.55±13.9                    | 0.712   |
| Infection history (positive/negative) | 20/35                        | 23/98                         | 0.013*  |
| Hematuria (positive/negative) | 39/16                        | 63/58                         | 0.019*  |
| SBP (mmHg)               | 129 (89-180)                 | 130 (88-212)                  | 0.886   |
| DBP (mmHg)               | 85 (62-130)                  | 86 (55-130)                   | 0.725   |
| Hemoglobin (g/L)         | 131 (82-192)                 | 130 (61-168)                  | 0.614   |
| ALB (g/L)                | 38.5 (11.3-51.8)             | 38.4 (10.1-52.4)              | 0.891   |
| BUN (mmol/L)             | 5.28 (2.85-34.89)            | 6.07 (2.4-39)                 | 0.033*  |
| SCr (μmol/L)             | 76 (29-851)                  | 82.4 (32-1133)                | 0.153   |
| eGFR (mL/min)            | 76.86 (63.3-164.24)          | 72.51 (4.29-174.69)           | 0.377   |
| Uric acid (μmol/L)       | 315 (149-559)                | 342.5 (145-850)               | 0.285   |
| Cystatin C (mg/L)        | 0.94 (0.4-5.24)              | 1.07 (0.6-5.47)               | 0.017*  |
| β2–MG (mg/L)             | 1.86 (0.27-10.88)            | 2.41 (0.01-18.75)             | 0.014*  |
| Proteinuria (g/24h)      | 1.23 (0.11-14.3)             | 1.57 (0.07-29.8)              | 0.254   |
| M0/M1                    | 46/9                         | 98/23                         | 0.673   |
| E0/E1                    | 43/12                        | 91/30                         | 0.668   |
| S0/S1                    | 33/22                        | 71/50                         | 0.869   |
| T0/T1/T2                 | 52/2/1                       | 95/15/11                      | 0.029*  |

Associations between deposition of SIgA and clinicopathological parameters

Table 2 compares the clinicopathological parameters between the SIgA-positive and SIgA-negative group. Patients in the SIgA-positive group had significantly a higher incidence of infection history (such as upper respiratory tract and/or gastrointestinal infections, including tonsillitis and diarrhea, P = 0.013) and hematuria (including both microscopic and macroscopic hematuria, P = 0.019), lower levels of serum cystatin C (P = 0.017), β2 microglobulin (P = 0.014) and blood urea nitrogen (P = 0.033), compared with the SIgA-negative group. No significant differences were detected in the levels of hypertension, hemoglobin, serum albumin, eGFR or proteinuria/24 h.

Patients in the SIgA-positive group had a significantly lower T-grade in the Oxford classification (P = 0.029) (Table 2, Fig. 2), compared with the SIgA-negative group. No significant differences were detected E, M and S in the Oxford classification.

Purification and identification of SIgA

A typical chromatogram of SIgA separated by jacalin agarose column is shown in Fig. 3. The peak A was the eluting peak, in which no IgA was detected by ELISA; peak B was the SIgA fraction, in which no IgG or IgM was detected by ELISA.

The purity and identity of the purified SIgA from the SIgA-positive group (P-SIgA) and healthy volunteers (N-SIgA) were confirmed and evaluated by SDS-PAGE and western blot, respectively, compared with commercial human SIgA (Fig. 4). Under reducing SDS-PAGE conditions, SIgA molecules in all pooled fractions were resolved into a SC, with heavy and light chains of predicted molecular weight 75,56 and 23 kDa, respectively (Fig. 4A). Both SC (Fig. 4B) and IgA (Fig. 4C) were detected in purified SIgA by western blot.
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**Proliferation of HRMCs induced by SIgA or pIgA**

In our study, P-SIgA-induced HRMCs proliferation was significantly higher compared with that induced by N-SIgA ($P<0.05$ at 12h and 24h, $P<0.001$ at 36h). Similarly, P-pIgA-induced HRMC proliferation was significantly higher compared with that induced by N-pIgA ($P<0.05$ at 12h and 24h, $P<0.001$ at 36h). Furthermore, P-pIgA-induced HRMC proliferation was significantly higher at 12, 24 and 36 h compared with that induced by P-SIgA ($P<0.05$ at 12h, $P<0.001$ at 24h and 36h, Fig. 5).

**Cytokines production by HRMCs cultured with SIgA or pIgA**

Fig. 6 shows the protein synthesis and mRNA expression of IL-6, IL-8, MCP-1, TGF-β1 and fibronectin in HRMCs cultured with SIgA or pIgA (200 µg/ml) from IgAN patients and healthy subjects. Both sources of SIgA or pIgA induced the upregulation of protein synthesis and mRNA expression of all cytokines tested from HRMCs except for TNF-α (data not shown).

SIgA and pIgA from patients induced higher protein synthesis of cytokines in stimulated cultured HRMCs when compared with SIgA and pIgA from healthy characters. P-SIgA increased protein synthesis of IL-6, IL-8, MCP-1, TGF-β1 and fibronectin at 24h by 2-, 1.6-, 1.9-, 1.3- and 1.5-fold respectively when compared with N-SIgA ($P<0.001$ for IL-6, IL-8, MCP-1, TGF-β1, $P<0.05$ for fibronectin). Under the same experimental
conditions, P-pIgA increased by 1.6-, 1.4-, 2-, 1.3- and 1.3-fold protein synthesis of IL-6, IL-8, MCP-1, TGF-β1 and fibronectin respectively when compared with N-pIgA at 24h (P<0.001 for all). Furthermore, P-SIgA-induced protein release of IL-6, IL-8 and fibronectin were significantly lower compared with that induced by P-pIgA (P<0.001 for all).

Similarly, SIgA and pIgA from patients induced higher mRNA expression of cytokines in stimulated cultured HRMCs when compared with SIgA and pIgA from healthy subjects. mRNA expression of IL-6, IL-8, MCP-1, TGF-β1 and fibronectin stimulated by P-SIgA was increased by 1.6-, 1.5-, 2-, 2.1- and 1.8-fold, respectively, compared with that by N-SIgA (P<0.001 for IL-6, MCP-1, TGF-β1 and fibronectin, P<0.05 for IL-8). Under the same experimental conditions, P-plgA increased by 2.3-, 1.4-, 2.1-, 2.4- and 1.5-fold mRNA expression of IL-6, IL-8, MCP-1, TGF-β1 and fibronectin, respectively when compared with N-plgA (P<0.001 for all). P-SIgA-induced mRNA expression of IL-6, IL-8 and fibronectin was significantly lower compared with that induced by P-plgA (P<0.001 for IL-6 and IL-8, P<0.05 for fibronectin).

**IL-6 is a direct target of miR-16**

Bioinformatic analysis using TargetScan and miRanda predicted that the 3′ UTRs of IL-6 contain binding sites for miR-16 (Fig. 7A). Expression of miR-16 was significantly downregulated in HRMCs stimulated by P-SIgA, compared with that stimulated by N-SIgA (P<0.001, Fig. 7B). To verify whether IL-6 is a direct target of miR-16, we used a Dual-Luciferase reporter system containing either wild-type or mutant 3′ UTRs of IL-6, and its
accuracy was identified by endonuclease digestion and DNA sequencing (Fig. 7C and D). Co-transfection with miR-16 significantly suppressed the luciferase activity of the reporter containing the wild-type 3′ UTR, and this did not happen in the mutant 3′ UTR (P<0.001, Fig. 7E). The result shows that IL-6 is a direct target of miR-16.

miR-16 regulates P-SIgA-stimulated IL-6 at the post-transcriptional level in HRMCs

Western blot showed that the protein level of IL-6 was decreased in the mimics group, compared with the blank and scramble groups (P<0.001, Fig. 8A). There were no differences in mRNA expression of IL-6 in the three groups (P>0.05, Fig. 8B). These results indicate that miR-16 negatively regulates IL-6 expression by directly binding to putative binding sites in the 3′ UTR. miR-16 mimics significantly suppressed P-SIgA-induced IL-6 at the post-transcriptional level in HRMCs.

Discussion

IgAN is recognized as the most common form of primary glomerulonephritis in developed countries, and remains an important cause of end-stage renal disease [24, 25]. However, the mechanism of IgAN is still unknown.

About 40% of patients with IgAN have recurrent episodes of macroscopic hematuria frequently preceded one or one day earlier by mucosal infections of the upper respiratory tract or digestive system [26]. SlgA is the most important antibody in mucosal immunity and is the immune systems first line of defense against pathogens [27]. After tonsillar stimulation, patients with IgAN, especially those suffering from macroscopic hematuria, had high levels of serum SlgA and elevated salivary levels of SlgA [28, 29]. High sera SlgA concentrations correlated with macroscopic hematuria in IgAN [26, 30]. Deposition of SlgA in the mesangium was also observed in patients with IgAN and the eluate of a nephrectomized transplanted kidney from a patient with IgAN contained a 120-fold accumulation of SlgA compared with IgA1 [2, 6, 27]. Taken together, these findings indicate mucosal immunity is closely associated with IgAN and that SlgA might play a pathogenic role in IgAN. Whether IgAN patients with
Fig. 6. Production of cytokines in HRMCs cultured with purified SIgA or plgA. The protein release of IL-6, IL-8, MCP-1, TGF-β1, and fibronectin from HRMC incubated for 12, 24 or 36 h and mRNA expression at 24 h are shown. Production of cytokines by HRMCs incubated with SIgA purified from IgAN patients (P-SigA) and healthy volunteers (N-SigA), plgA isolated from IgAN patients (P-plgA) and healthy subjects (N-plgA), at 200 µg/ml. The table below each graph depicts statistical analysis between groups using one-way analysis of variance and LSD test. Expression of mRNA is expressed as $2^{-\Delta\Delta Ct}$. Results are expressed as mean ± SD. (* $P<0.001$, # $P<0.05$, NS: $P>0.05$).
or without IgA deposition have similar clinical and pathological characteristics is unknown. In the current study, of 176 IgAN patients enrolled, 55 (31.25%) with IgA deposition had similar clinical manifestations including hypertension, eGFR, proteinuria/24 h and pathological glomerular changes such as M, E, S in the Oxford classification compared with patients without IgA deposition. This indicated IgA might have a similar pathogenic role to that of IgA in the progress of IgAN. However, the IgA-positive group had a higher incidence of infection ($P = 0.013$) and hematuria ($P = 0.019$), compared with the IgA-negative group.

Fig. 7. IL-6 is a direct target of miR-16. IL-6 was identified as a target of miR-16 in HRMCs. A. The putative miR-16 binding sequences for the IL-6 3' UTR. B. Expression of miR-16 in P-HRMCs and N-HRMCs (P-HRMCs: HRMCs stimulated by P-SigA, N-HRMCs: HRMCs stimulated by N-SigA; *$P<0.001$). C. Electrophoresis of double endonuclease digestion (XhoI and XbaI): recombinant vector was divided into IL-6 3' UTR (256 bp) and vector pmirGLO (7.3kb). D. DNA sequencing. Wt 3' UTR: wild type of IL-6 3' UTR, Mut 3' UTR: mutant of IL-6 3' UTR. E. Dual-luciferase reporter co-transfection with miR-16 and wild-type or mutant 3' UTRs of IL-6. The reporter containing the wild-type 3' UTR significantly suppressed luciferase activity. *$P<0.001$ compared with the control group.

Fig. 8. Endogenous regulation of miR-16 on IL-6 in HRMCs. HRMCs were transfected with mimic miR-16 and induced with P-SigA. Over-regulation of miR-16 inhibited expression of IL-6 at the post-transcriptional level. Blank: nontransfected blank group; Scramble: transfected with scrambled miR-16; Mimic: transfected with miR-16 mimic. A. Protein level of IL-6 in supernatant of HRMCs detected by Western blot in three groups; GAPDH as a reference. *$P<0.001$ compared with the control group. B. Relative expression of IL-6 mRNA in three groups. $P>0.05$ in three groups.
This further confirms the important role of mucosal immunity in patients with IgAN, especially those with SIgA deposition.

Aberrant glycosylation of IgA1 plays a crucial role in the pathogenesis of IgAN. It is deposited in the renal mesangium and stimulates mesangial cells or activates complement pathways to induce renal damage [31, 32]. SIgA is secreted by B cells in mucosal cells, where it enters the circulation and is finally deposited in the mesangium. Thus, could SIgA have similar biological effects to IgA in renal injury in IgAN? To answer this question, we measured the impact of SIgA from SIgA deposition patients on mesangial cells.

An important point is how to isolate SIgA from patients with IgAN? To date, this has not been reported to the best of our knowledge. SIgA mainly spreads in exocrine secretions such as saliva, intestinal fluid, respiratory tract mucus and breast milk, especially colostrum. Human tonsil tissues have a close relationship with IgAN and salivary SIgA acts as a marker for immune status [33, 34]. The level of SIgA in saliva provides much information about the functional status of the entire mucosal immune system [34]. In addition, its concentration in saliva is high (~35–81 μg/ml) but low in serum (~9–18 μg/ml) in adults [6, 34-36]. Moreover, previous studies have shown underglycosylated IgA molecules produced by tonsils could be one origin of glomerular IgA in patients with IgAN [33, 37, 38]. Therefore, we isolated SIgA from saliva using jacalin affinity chromatography. This is believed to be the first report of purified SIgA from IgAN patients.

Previous studies showed cytokines such as IL-6, IL-8, MCP-1, TGF-β1, fibronectin and vascular endothelial growth factor were important in the pathogenesis of IgAN [12, 39-41]. P-IgA from patients with IgAN stimulated mesangial cell proliferation and produced these cytokines at higher levels than plgA isolated from healthy people [12, 31, 40-42]. IL-6, IL-8 and MCP-1 induce the proliferation of mesangial cells and have a proinflammatory effect in glomerular injury of IgAN [12, 43, 44]. TGF-β1 and fibronectin are important for the accumulation of extracellular matrix proteins and promote fibrogenesis in IgAN [40, 43, 45, 46]. In the present study, SIgA also induced mesangial cell proliferation and induced production of IL-6, IL-8, MCP-1, TGF-β1, and fibronectin. The effects of SIgA isolated from IgAN patients were greater compared with those of SIgA from healthy persons. Our results indicated SIgA might have a pathogenic role in IgAN, perhaps by inducing the production of cytokines such as IL-6, IL-8, MCP-1, TGF-β1, and fibronectin to induce kidney injury.

In the present study, compared with SIgA-negative group (IgA deposition), the SIgA-positive group had a higher incidence of hematuria and lower T scores for the Oxford classification. P-SIgA induced lower HRMC proliferation and production of IL-6, IL-8 and fibronectin than P-plgA induced, which indicated that the biological effects of SIgA on HRMC might be weaker than those of plgA. A study published this year reported that compared with IgAN patients without hematuria, those with hematuria had lower pathological scores such as M, S and T in the Oxford classification, and better renal survival [47]. Based on our findings, this observation may be associated with different IgA mesangial deposition in IgAN. However, whether it is true still needs further exploration.

How does SIgA mediate these biological effects? Is it through the regulation of miRNAs? miRNAs are short (approximately 22 bases) noncoding RNA molecules that post-transcriptionally regulate gene expression through incomplete base pairing with the 3’ UTR of target mRNAs [15]. They have been estimated to regulate up to 30% of all human genes and to control a variety of cellular processes [48]. In IgAN, there are few reports about the regulation of miRNAs in mesangial cells. miR-16 is predicted to be a critical regulator in mucosal immunity [23]. It could induce rapid degradation of cytokine and chemokine RNAs such as TNF-α, IL-8 and IL-6 mRNAs, which contain AU-rich elements in their 3’ UTRs [49, 50]. It is not known whether miR-16 downregulates the inflammatory effects of SIgA in mesangial cells. We found that expression of miR-16 in HRMCs stimulated by P-SIgA was significantly lower than that stimulated by N-SIgA. We also verified that the target gene of miR-16 is IL-6. Overexpression of miR-16 could significantly decrease the protein level of IL-6 in HRMCs stimulated by P-SIgA (P<0.01), which indicates that miR-16 significantly suppresses P-SIgA-induced IL-6 at the post-transcriptional level in HRMCs. Therefore, we
speculate that SIgA from IgAN patients could cause miR-16 to regulate release of IL-6 in HRMCs.

We found that the biological effects of SIgA on mesangial cells are different from those of pIgA, and SIgA can also stimulate HRMCs, inducing their proliferation and producing proinflammatory cytokines. Our findings provide a new explanation that the molecular mechanism of IL-6 production after SIgA stimulation in mesangial cells is regulated by miR-16. These results indicate an important role and partial mechanism for SIgA in the pathogenesis of IgAN.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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