Expression of immunoglobulin A in human mesangial cells and its effects on cell apoptosis and adhesion

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Abstract. IgA nephropathy (IgAN) is characterized by predominant IgA deposition in the glomerular mesangium. It has been considered that the deposited IgA is synthesized by B cells, although recent reports have suggested the implication of other cell types. Therefore, the present study investigated whether glomerular mesangial cells could produce IgA by themselves. Semi-quantitative reverse transcription-polymerase chain reaction, and immunostaining analysis revealed that the IgA protein and gene transcripts were expressed in primary human renal mesangial cells (HRMCs). Furthermore, the IgA heavy chain ($\alpha1$ and $\alpha2$) and the light chain (κ and λ) were localized in the cytoplasm or were located on the cell membranes of human mesangial cells (HMCs). Mass spectrometry results indicated that Ig $\alpha1$ and Ig $\alpha2$ were secreted in the culture media of HMCs. The transcripts of Ig α, Ig κ and Ig λ constant regions were detected. The predominant rearrangement pattern of the variable region of Ig κ, was Vκ3-20*01/Jκ1*01 in HMCs and Vκ1-12*01/Jκ4*01 in HRMCs. In addition, knockdown of Ig $\alpha1$ expression by small interfering RNA (siRNA) inhibited cell adhesion and promoted apoptosis. Our findings

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demonstrate that HMCs can express IgA, and that this expression is associated with cell functions, which may contribute to the deposition of IgA in patients with IgAN.

Introduction

Human mesangial cell (HMC) proliferation and expansion occurs in major glomerular diseases and is the main feature of IgA nephropathy (IgAN) (1). It is generally considered that the immune complexes containing IgA are found in the glomerular mesangium, and that IgA1 is secreted by B lymphocytes, mediated by the process of glycosylation and over aggregation. The abnormal IgA1 can be recognized by anti-glycan auto-antibodies of the IgA1 and/or IgG isotype, resulting in formation of circulative immune complexes (CIC) (2,3). The pathogenic CIC deposits in the glomerular mesangium can promote resident mesangial cells to secrete proinflammatory factors, initiating glomerular injuries (4-6).

Classical immunology considers that differentiated B cells are the unique source of immunoglobulins (Igs). However, this theory has been challenged over the past decade by increasing evidence reporting that Igs could be expressed in cancer cells. Qiu and Yang initially reported the existence of Ig-like protein in malignant tumor cells in 1996 (7,8). Later studies by Kimoto and Zheng *et al* have shown that Igs transcripts are expressed in human carcinoma cell lines (9), and in human epithelial carcinoma cell lines (10). Qiu *et al* also has reported IgG secretion by epithelial cancer cells, and demonstrated that its function is to promote growth and survival of tumor cells (11). Subsequently, Igs were found to be widely expressed in many types of cancer cells, including breast cancer, colon cancer, lung carcinomas, nasopharyngeal carcinoma, abnormal cervical epithelial cells and oral epithelial tumor cells (12-16). Unlike B-cell-derived Igs, which are the key molecules for humoral immune responses, cancerous Igs are associated with various cell functions, such as cell survival, proliferation, transformation, metastasis and carcinogenesis (11,13,17-22).

Besides the cancer cells, there is growing evidence showing that normal cells could also express Igs. Huang *et al* reported that several types of Igs are expressed in normal cells, including IgG expression in brain neurons with classic V-(D)-J gene rearrangements (23), Ig μ gene expression and

rearrangement in myeloid cells (24), Ig gene expression and rearrangement in germ cells (25), mammary gland (26) and hematopoietic stem/progenitor cells (27). Kang *et al* revealed the LOX-1 dependent overexpression of Ig κ in cardiomyocytes in response to angiotensin II (AngII) (28). Previous results detected the IgG expression in the eye (29), and the IgG, IgA, IgM expression in the liver (30) and in the hippocampus (31). These findings demonstrated that normal cells could express proteins and mRNA transcripts of the Ig's heavy chains, light chains, and enzymes required for V(D)J recombination, suggesting a significant role in maintaining the organs' micro-environment, and regulating the development and function of cells.

In the present study, we have confirmed that IgA is expressed in primary human renal mesangial cells (HRMCs) and in the HMCs, and investigated its potential role on cell apoptosis and cell adhesion.

Materials and methods

Cell culture. Primary HRMCs (Sciencell Research Laboratories, Carlsbad, CA, USA) were cultured in mesangial cell medium (MCM) solution containing 2% FBS, 1% mesangial cell growth supplement, and 1% penicillin/streptomycin. The materials to culture HRMCs were purchased from the Sciencell Research Laboratories and cultured according to the manufacturer's protocol. Cells were maintained in serum-free medium for 48 h prior to harvesting. Cells were used at passage nos. 4 to 6.

The HMC line, C2M12, which retains many of the morphological and physiological features of the normal HMCs (32,33), was kindly donated by Professor Youfei Guan (Department of Physiology and Pathophysiology, Peking University Health Science Center, Peking, China). These cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Biological Industries USA, Inc., Cromwell, CT, USA), 1% insulin transferrin selenium-A supplement (ITS-A; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were sub-cultured when reaching 90% confluency with 0.05% trypsin containing 1 mM EDTA for 20 sec at 37°C. AngII and staphylococcus (SAC; Sigma-Aldrich, St. Louis, MO, USA) were used to stimulate the HMCs.

Cell cycle synchronization. Cell cycle synchronization of the HMCs was performed following the double thymidine block protocol described by previous studies (34,35). Briefly, HMCs were seeded on 10 cm culture dishes at a density of $1x10^5$ cells per dish. In order to collect cells arrested at G1/S phase, the cell culture was grown until it reached confluence of 50%, then arrested with 2 mmol/l thymidine in complete culture media for 12 h, washed twice with phosphate-buffered saline (PBS), and recovered in fresh complete culture media for 12 h, followed by a second arrest with 2 mmol/l thymidine for another 12 h. After the second arrest, the supernatant was replaced by fresh complete culture media to recover the cells. A sample of each cell culture was collected on cover slips every 2 h after the second cell cycle release.

Cell cycle assay. Cell cycle progression was assessed by flow cytometry based on the DNA content of cells (36). DNA content of cells at distinct phases of the cell cycle (G0/G1, S, and G2/M phase) was analyzed using propidium iodide (PI) staining. HMCs were harvested and washed twice in cold PBS by centrifugation at 800 x g for 5 min. Cells were then suspended in 100 μl ice-cold PBS at a density of at least $2x10^4$ cells per tube. 3 ml of ice-cold 70% ethanol was gradually added to the cell suspension for fixation. The suspended cells were incubated at 4°C overnight, then filtered through a 48 μm filter screen, spun at 1,500 x g for 5 min and washed twice with ice-cold PBS to remove traces of ethanol. RNase (0.5 mg/ml) was added to degrade RNA at 37°C for 30 min. After washing twice with 300 μl ice-cold PBS, the cells were suspended in 300 of 50 μg/ml PI staining solution to stain the nuclei and incubated at room temperature for 5 min in the dark. The cell cycle data for individual samples was acquired using the BD LSRFortessa™ flow cytometer equipped with BD FACSDiva™ software (BD Biosciences, San Diego, CA, USA) and analyzed using ModFit LT™ software (Verity Software House, Topsham, ME, USA).

Immunofluorescence. For indirect immunofluorescence staining (IF), HMCs and HRMCs were cultured on cover slips and fixed in cold acetone for 5 min. After washing three times with PBS, the slides were blocked with 5% BSA (Invitrogen; Thermo Fisher Scientific, Inc.) (diluted with PBS) for 30 min at room temperature and incubated with the primary antibody (diluted with PBS) at 4°C overnight. Mouse anti-human Ig α1, Ig α2 antibodies (Southern-Biotech, Birmingham, AL, USA), mouse anti-human monoclonal Ig κ, Ig λ antibodies (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) were used as the primary antibody; PBS was used as a blank control. After removing the unbound antibodies by washing in PBS for three times, the slides were incubated with goat anti-mouse IgG antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd.) and labeled with fluorescein isothiocyanate (FITC) for 1 h at room temperature in dark. For direct immunofluorescence staining, the slides were incubated with the mouse anti-human Ig α-FITC (Zhongshan Golden Bridge Biotechnology Co., Ltd.) in the dark overnight at 4°C. After washing another three times, the slides were incubated with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) for 2 min at room temperature. Fluorescent signals were detected with a Confocal Laser Scanning microscopy FV1000 (Olympus, Tokyo, Japan).

Semi-quantitative reverse transcription-polymerase chain reaction (SqRT-PCR). Total RNA of cultured cells was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the concentration was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Then 1.5 μg of total RNA was reverse-transcribed to cDNA using the GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed with the primers targeting constant regions of Ig α, Ig κ, Ig λ (Ig Cα, Ig Cκ, Ig Cλ) and nested PCR was performed with external primers at the first round and internal primers at the second round targeting variable region of Ig κ (Ig Vκ). The sequences of primers and reaction conditions are listed in Tables I and II.

Table I. Sequences of polymerase chain reaction primers used in this study.

Gene name	Primer	Primer sequence 5'-3'	Product length (bp)
Igα constant region (Ig Cα)	Forward	ACCATGCAGGAGAAGGTGTC	340
	Reverse	TCACTTGCACTGCTGCCTAC	
Igκ constant region (Ig Cκ)	Forward	TGAGCAAAGCAGACTACGAGA	231
	Reverse	GGGGTGAGGTGAAAGATGAG	
Igλ constant region (Ig Cλ)	Forward	GGGACCAAGCTCACCGTCCTAG	316
	Reverse	TCTTCTCCACGGTGCTCCCTTC	
Igκ variable region (Ig Vκ)	External forward	GACATCGAGCTCACCCAGTCTCC	360-380
	External reverse	CGGGAAGATGAAGACAGATGGTGC	
	Internal forward	GAAATTGAGCTCACGCAGTCTCCA	340-360
	Internal reverse	TGGTGCAGCCACAGTTCGTT	
β-actin	Forward	AGAGCTATGAGCTGCCTGAC	121
	Reverse	AATTGAATGTAGTTTCATGGATG	

Table II. Reaction conditions of polymerase chain reaction used in this study.

Gene name	Initial denaturation (˚C/min)	Denaturation (˚C/sec)	Annealing (˚C/sec)	Extension (˚C/sec)	Cycle number	Extension (˚C/min)
Ig Cα	94/4	94/30	62/30	72/30	35	72/10
Ig Cκ	95/4	95/30	50/30	72/30	35	72/10
Ig Cλ	95/4	95/30	56/30	72/30	35	72/10
Ig Vκ (External reaction)	94/5	94/30	60/30	72/30	3	-
		94/30	58/30	72/30	3	-
		94/30	56/30	72/30	3	-
		94/30	54/30	72/30	3	-
		94/30	52/30	72/30	3	-
		94/30	50/30	72/30	3	-
		94/30	48/30	72/30	20	72/7
Ig Vκ (Internal reaction)	94/5	94/30	60/30	72/30	35	72/7
β-actin	94/5	94/30	56/30	72/30	25	72/7

Amplification products were separated in a 1% agarose gel by electrophoresis, including a 100 bp DNA ladder (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The amplified DNA fragments were identified by their molecular mass, under ultraviolet light observations. Human peripheral blood mononuclear cells (PBMCs) were used as the positive control. The peripheral blood was obtained from healthy donors. PBMCs were isolated from 5 ml peripheral blood using two-step discontinuous Ficoll/Hypaque (Second Chemistry Factory, Shanghai, China) density gradient centrifugation. The white gradient layer containing PBMCs was recovered and washed with 0.01 M PBS, and the isolated PBMCs used immediately for total RNA extraction (37).

Analysis of gene rearrangement. PCR products of Ig Vκ were cloned into a pGEM-T Easy Vector (Promega) and transfected into the competent *E. coli* cell line TOP10 [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. The transcripts of individual clones were amplified. After DNA sequencing with an ABI 3730XL Genetic Analyzer (Applied Biosystems;

Thermo Fisher Scientific, Inc.) which was performed by Invitrogen; Thermo Fisher Scientific, Inc., the variable sequences were compared with the published sequences of the germline gene segments using the BLAST tool of the National Center for Biotechnology Information (NCBI).

Western blot analysis. Cultured cells were harvested and washed twice with cold PBS, then re-suspended in TSD lysis buffer (TSD lysis buffer, 1% SDS; 50 mmol/l, pH 7.5 Tris-HCL, 50 mmol/l DTT), sonicated for 1 min, and lysed for 30 min at room temperature. The protein concentration of the cell lysate was calculated with a BCA kit (Applygen Technologies Inc., Beijing, China). After centrifugation at 12,000 x g for 10 min at 4˚C, 5X loading buffer was added to the lysate, boiled at 100˚C for 5 min, and the samples were immediately used for western blot analysis. The proteins in the culture supernatant were precipitated with 50% ammonium sulfate, centrifuged at 12,000 x g for 15 min and then dissolved in PBS. The collected fraction was filtrated with the AmiconR Ultra-0.5 Centrifugal Filter Devices (EMD

Millipore, Billerica, MA, USA) to remove the ammonium sulfate. The measurement of protein concentration in the culture supernatant was performed with the same method used as for the cell lysate. Free Ig in the human serum and the cultural medium were used as control.

The protein samples were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were incubated with rabbit anti-IgA anti-body (1:1,000), mouse anti-IgA1 antibody (1:1,000), mouse anti-IgA2 antibody (1:1,000), rabbit anti-Ig κ antibody (1:10,000), and rabbit anti-Ig λ antibody (1:50,000). The above antibodies were purchased from Abcam (Cambridge, UK). All the membranes were washed three times with TBST for 10 min before incubated with secondary antibodies for 1 h at room temperature. Goat anti-rabbit IgG-IRDyeTM800CW (1:10,000 and goat anti-mouse IgG-IRDyeTM680CW (1:10,000; both from LI-COR Biosciences, Lincoln, NE, USA) were used as secondary antibodies. Immunoreactivity was observed with the Odyssey Infrared imager (LI-COR Biosciences).

IgA1 purification and mass spectrometry. After the HMCs were cultured in RPMI-1640 with 2% FBS for 48 h, the culture supernatant was collected as described above. IgA1 was purified according to the manufacturer's instructions of jacalin-sepharose (BioVision, Milpitas, CA, USA). After precipitation of the proteins, the pellet was dissolved in PBS and filtrated with the AmiconR Ultra-0.5 Centrifugal Filter Devices (EMD Millipore) to remove the ammonium sulfate and elution buffer. The purified proteins were separated by 10% SDS PAGE, detected by western blot analysis as described above, and further analyzed by mass spectrometry in the Beijing Protein Innovation Co., Ltd. (Beijing, China).

Cell stimulation with AngII. HMC were seeded on 10 cm culture dishes. When the sub-cultured HMC reached 70% confluency, cells were cultured in RPIM-1640 containing 0.5% FBS overnight, followed by treatment with 10^{-8} mol/l AngII for 24 h or with SAC (1:1,000) for 48 h. A sample from each cell culture was placed on cover slips for immunostaining and the remaining cells were collected for western blot analysis, as described above.

Transfection of cultured HMCs with small interfering RNA (siRNA). Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the transfection with siRNA. siRNAs directed against different regions of the constant region of the Ig α1 heavy chain (siRNA-1, siRNA-2 and siRNA-3), against GAPDH (positive control, PC) and against the nonspecific, scrambled, control siRNA [negative control (NC)], were designed by GenePharma Company (Shanghai, China) and the sequences are listed in Table III. HMCs were seeded onto 12-well culture plate (2×10^4 cells/well) in mesangial cell culture media and grown overnight. HMCs were then transfected with each of 50 nmol/l siRNA mixed with Lipofectamine reagent in Opti-mem medium (Invitrogen; Thermo Fisher Scientific, Inc.). PBS was added to the control group. HMCs were harvested after transfection for 48 h and used for western blot analysis.

Table III. Sequences of siRNA used in this study.

siRNA	Direction	Sequence (5'-3')
siRNA-1	Forward	GCUCUUAGGUUCAGAAGCGTT
	Reverse	CGCUUCUGAACCUAAGAGCTT
siRNA-2	Forward	GGAACCAUGGGAAGACCUUTT
	Reverse	AAGGUCUUCCCAUGGUUCCTT
siRNA-3	Forward	GCCUUCACACAGAAGACCATT
	Reverse	UGGUCUUCUGUGUGAAGGCTT
Positive control	Forward	UGACCUCAACUACAUGGUUTT
	Reverse	AACCAUGUAGUUGAGGUCATT
Negative control	Forward	UUCUCCGAACGUGUCACGUTT
	Reverse	ACGUGACACGUUCGGAGAATT

siRNA, small interfering RNA.

Table IV. Rearrangement patterns of Ig κ variable region transcripts.

Name	Clone no.	Vκ	Jκ	Identity%
PBMC (n=16)	1	Vκ1-27*01	Jκ1*01	90.2
	1	Vκ1-27*01	Jκ4*01	96.1
	1	Vκ1-39*01	Jκ1*01	90.8
	6	Vκ1-39*01	Jκ4*01	86.9-97.6
	1	Vκ1-39*01	Jκ3*01	97.9
	1	Vκ1-39*01	Jκ5*01	93.3
	1	Vκ1-16*02	Jκ4*01	95.5
	2	Vκ1-33*01	Jκ4*01	91.3
	1	Vk4-1*01	Jk4*01	96.6
HMC (n=7)	7	Vκ3-20*01	Jκ1*01	94.4
HRMC (n=8)	2	Vκ3-20*01	Jκ1*01	92.7-94.4
	6	Vk1-12*01	Jk4*01	94.4-94.7

PBMC, peripheral blood mononuclear cell; HMC, human mesangial cell; HRMC, human renal mesangial cell.

Cell apoptosis assay. After transfection for 48 h, cells were collected with 0.05% trypsin solution and harvested by centrifugation at 800 x g for 5 min. The harvested cells were washed twice with cold PBS. According to the manufacturer's protocol, 1×10^6 cells were suspended in 100 μl of 1X Annexin V binding buffer and stained with 5 μl of Annexin V-FITC and 5 μl of 7-AAD (both from BD Biosciences) in the dark for 15 min at room temperature. After adding another 400 μl binding buffer and filtrating through a 48 μm filter, cell apoptosis was measured by flow cytometry (BD Biosciences).

Cell adhesion assay. Cell adhesion rate was analyzed by the Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After siRNA transfection for 48 h, 3×10^4 cells were re-suspended in culture media and 100 μl were
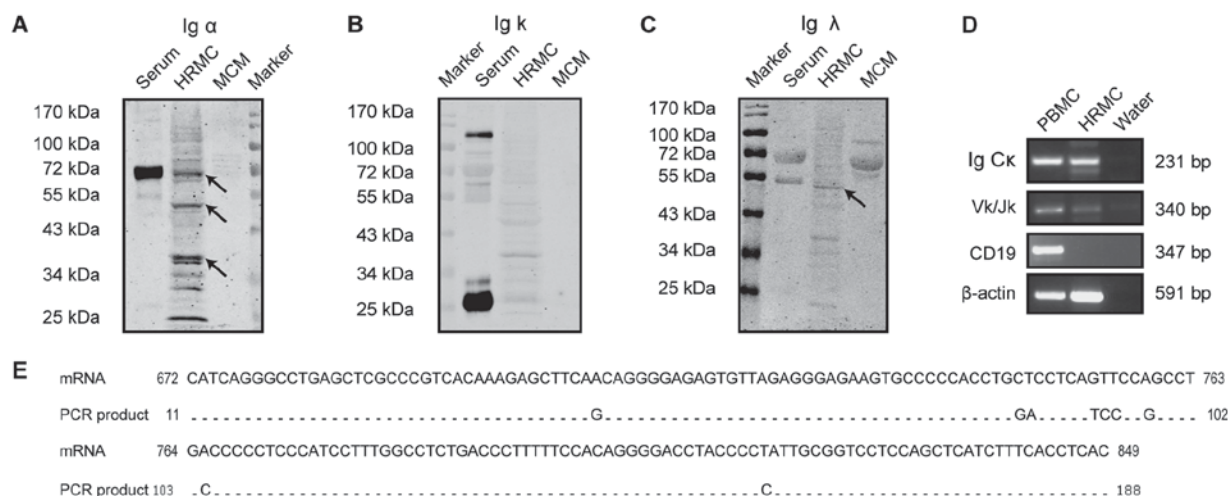
Figure 1. IgA expression in HRMCs. (A-C) Ig α and Ig λ bands detected in the primary human renal mesangial cell lysate and the human serum as a positive control; arrows indicate immunoreactivity against Ig α and Ig λ; (D) Ig κ constant region (Ig Cκ) gene transcript (231 bp) and variable region (Vκ/Jκ) in HRMCs detected by SqRT-PCR; (E) alignment of the SqRT-PCR product sequences from HRMCs with Ig Cκ mRNA sequences from NCBI database (Gene Bank, Y14736.1). Negative control: PCR reaction mixture with water; CD19: B lymphocyte marker. HRMC, human renal mesangial cell; SqRT-PCR, semi-quantitative reverse transcription-polymerase chain reaction; NCBI, National Center for Biotechnology Information; MCM, mesangial cell medium; PBMC, peripheral blood mononuclear cell.

aliquoted in each well of a 96-well plate and incubated at 37˚C for 1 h. 3 wells of each group were washed gently three times with PBS, and 100 μl of fresh culture media with 8 μl of CCK-8 reagents were added. In order to analyze the total cell concentration, CCK-8 was directly added to 3 different unwashed wells. After incubation for 3 h at 37˚C, concentration was determined by measuring absorbance at 450 nm using a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The cell adhesion rate was calculated as follows:

$$\text{Cell adhesion rate} = \frac{OD_{washed} - OD_{blank}}{OD_{unwashed} - OD_{blank}}$$

Statistical analysis. Data was expressed as the means ± standard deviation and analyzed using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). The differences between experimental groups were analyzed with one-way analysis of variance, followed by a Least Square Difference multiple comparison test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

IgA expression in primary HMCs. In this study, the *in vivo* IgA expression in HRMCs was investigated. Western blot analysis of the lysed HRMCs demonstrated that Ig α was present not only at a size of 72 kDa, which was consistent with the positive control in the serum, but also at 53 and 38 kDa (Fig. 1A), indicating that the α chains in the cytoplasm might be truncated or are at different synthesis stages. There was no obvious band detected for Ig κ (Fig. 1B). A positive band for Ig λ was detected at 55 kDa which is similar to the molecular weight of a dimer and was consisted with the size of the positive controls in the serum (Fig. 1C). The absence of a band in the MCM eliminates the possibility of IgA heavy chain and light chain expression in the culture medium. In addition, RT-PCR revealed Ig Cκ and Ig Vκ transcripts' expression in

HRMCs (Fig. 1D). Further sequencing of the PCR products showed 95% sequence similarity between the Ig Cκ collected from the HRMCs and the published sequence obtained from plasma cells in the NCBI database (Gene Bank, Y14736.1) (Fig. 1E). The predominant Vκ/Jκ rearrangement pattern was Vκ1-12*01/Jκ4*01, which is different to the transcripts' pattern observed in the PBMCs (Table IV). The negative expression of CD19 indicated that there was no B cell contamination in the HRMCs. The constant region and the Ig Vκ transcripts were strongly detected, suggesting an IgA expression in the HRMCs.

IgA expression in HMC line. IgA expression in mesangial cells was further confirmed in the HMC cell line with several methods. Immunofluorescence staining was positive for Ig α, Ig κ, Ig λ in the mesangial cytoplasm (Fig. 2A). Similar to a previous study which had reported the absence of IgA in the FBS (38), the FBS was negatively stained with rabbit anti-human Ig α, κ and λ antibodies, indicating that FBS could not interfere with the results. Western blot analysis of the HMCs lysates displayed similar positive bands for Ig α at 53 and 38 kDa, and for Ig λ at 55 kDa, but negative results for Ig κ, which corresponds with the results obtained in HRMCs (Fig. 2B), further supporting the IgA expression in mesangial cells.

Ig gene rearrangement and transcription is a prerequisite for Ig expression. To confirm the fact that IgA was synthesized in HMCs, we further explored the transcripts of Ig α, Ig κ and Ig λ by examining the mRNA expression of the constant regions of Ig α, Ig κ, Ig λ and the variable region of Ig κ in the HMCs (Fig. 2C). The alignment of the sequences of the RT-PCR products with those of the published Ig Cα1, Ig Cα2, Ig Cκ and Ig Cλ mRNA sequences in the NCBI database (Gene Bank, BC016369.1, BC073765.1, Y14736.1, X57823.1) demonstrated a sequence similarity of 99, 97, 98 and 97%, respectively (Fig. 3). The DNA sequencing of the Vκ PCR products showed that the predominant rearrangement was Vκ3-20*01/Jκ1*01, which was different from the rearrangements in HRMCs,
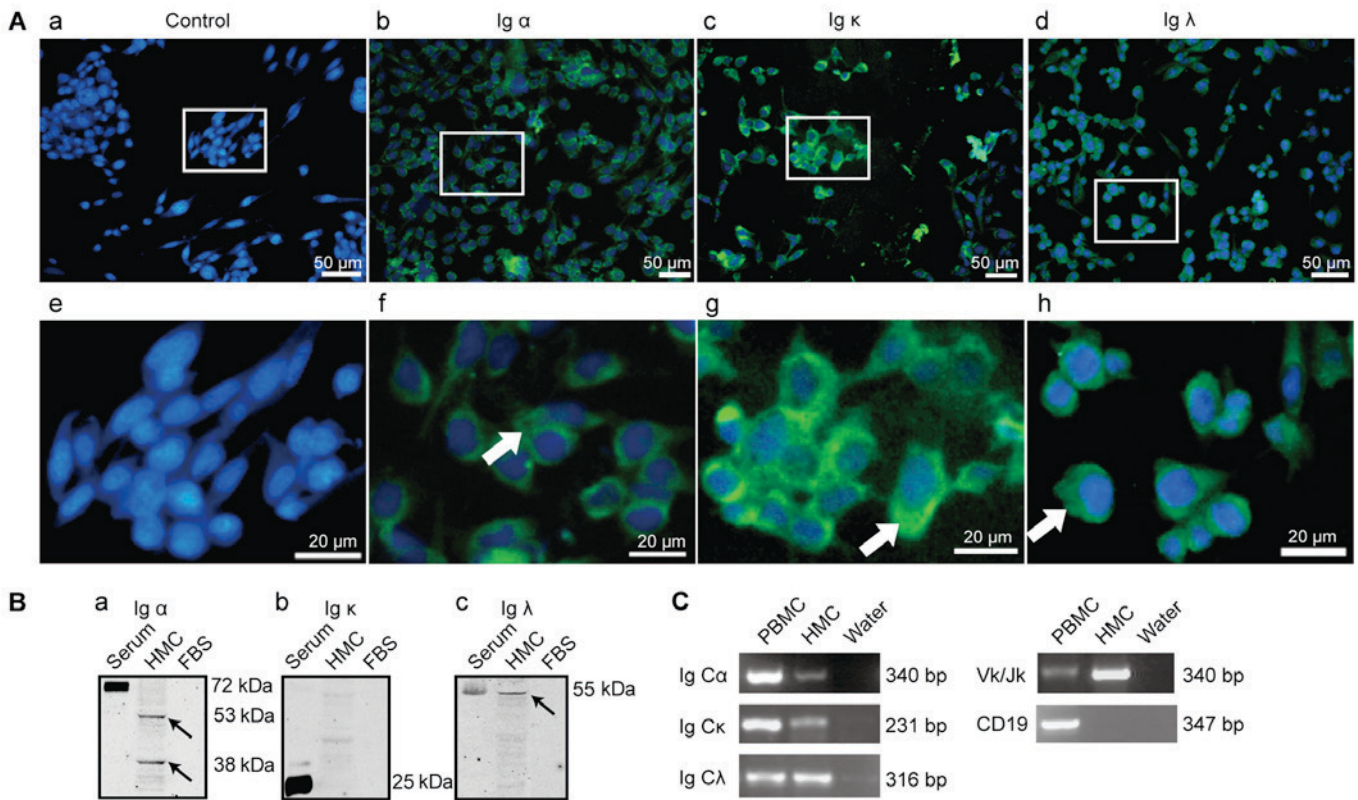
Figure 2. IgA expression in HMCs. (A) Positive immunofluorescence staining of Ig α (b and f), Ig κ (c and g) and Ig λ (d and h) in HMCs, (a and e) were the blank controls with PBS replacing primary antibodies; Green, antibody staining; blue, nuclear staining by DAPI; scale bar, 50 μm (upper), 20 μm (lower); (B) Ig α (a), Ig κ (b), Ig λ (c) detected in human glomerular mesangial cell line lysates and human serum as a positive control; arrows indicate immunoreactivity against Ig α and Ig λ; (C) Ig α heavy chain constant region (Ig Cα), Ig κ light chain constant region (Ig Cκ), Ig λ light chain constant region (Ig Cλ), and Ig κ variable region (Vκ/Jκ) transcripts correspond to the 340, 231, 316, and the 340 bp SqRT-PCR products, respectively. HMC, human mesangial cell; PBS, phosphate-buffered saline; SqRT-PCR, semi-quantitative reverse transcription-polymerase chain reaction; PBMC, peripheral blood mononuclear cell as positive control; negative control: PCR reaction mixture with water; CD19, The B lymphocyte marker.

and less diverse than the transcripts from PBMCs (Table IV). The HMC-derived Ig Vκ rearrangement sequence has been submitted to the GenBank database (GenBank accession no. KX443559).

Dynamic expression of IgA in HMCs during cell cycle and IgA secretion. Double thymidine (TdR) block model was used to achieve HMCs synchronous growth and the cells were harvested every 2 h to detect the IgA expression at different phases of the cell cycle. The cell cycle phase was determined by flow cytometry and demonstrated that HMCs entered S phase at around 2 h, G2/M phase from 4 to 6 h, G0/G1 phase from 8 h to 10 h, and re-entered into S phase 12 h later (Fig. 4A).

Consistent with the cell cycle phases, we detected dynamic expressions of Ig α1 (Fig. 4B and C) and Ig α2 (data not shown). The immunostaining results showed that Ig α1 expression was gradually increased from S phase (2 h), then reached highest levels at the G2/M phase (4-6 h), decreased after the G0/G1 phase (10 h) and increased in S phase (12 to 24 h) again. Different protein expression levels were also

detected in the cell lysates by Western blot analysis (Fig. 4D and E). The trend of the Ig α heavy chain expression at 4 and 10 h by Western blot was similar with that by IF staining. The α chain displayed a differential localization pattern and expression levels during the 24 h observation period after synchronization. These changes were in accordance with the cell G, S and M phases, indicating that the IgA heavy chain may be associated with cell growth, proliferation and division.

To find out whether HMCs could secrete IgA, we purified Ig α1 from the culture supernatant using jacalin-sepharose which binds to human IgA1 with high specificity. The size of the eluted protein was 65 kDa, according to the anti-human Ig α and the Ig α1 antibodies staining, which corresponds to the molecular size for the Ig α heavy chain (Fig. 4F). Mass spectra results showed that there was high homology between the amino acid sequences of the band and those of the Ig α1 and Ig α2 constant regions published in the NCBI database (GenBank, CAC20453.1, AAB30803.1) (Fig. 5).

Up-regulation of IgA in HMCs by AngII. We utilized AngII, endogenous pro-inflammatory factor, to examine the effects

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A   mRNA          1368 GCGCAGTATGCTGGTCACAGCGAAGGTGGTGGTGCCCTGGCTGGGCTCCTGCCGGGATGCCCAAGTCAGGTACTTCTCGCGGGGCAGCTC 1279
    PCR product   15   ........................................................................................ 104
    mRNA          1278 CTGTGACCCCTGCAGCCAGCGAACCAGCACGTCCTTGGGGCTGAAGCCGCGTGCCAGGCACGTCAGCGTCACCAGCTCGTTCAGGGCCAG 1189
    PCR product   105  ....................................A................................................... 194
    mRNA          1188 CTCCTCCGACGGCGGCGGCAGCAGGTGGACCTCGGGCCGGAATGTGTTTCCGGATTTTGAGAGGGTGGCGGTTAGCGGGGTCTTGGACTC 1069
    PCR product   195  ........................................................................................ 284
    mRNA          1068 GGGGTAGGCAGCAGTGCAAGTGAA 1075
    PCR product   285  ........................ 308

B   mRNA          1295 GCGCAGTATGCTGGTCACAGCGAAGGTGGTGGTGCCCTGGCTGGGCTCCTGCCGGGATGCCCAAGTCAGGTACTTCTCGCGGGGCAGCTC 1206
    PCR product   15   ........................................................................................ 104
    mRNA          1205 CTGTGACCCCTGCAGCCAGCGAACCAGCACGTCCTTGGGGCTGAAGCCGCGTGCCAGGCACGTCAGCGTCACCAGCTCGTTCAGGGCCAG 1116
    PCR product   105  ....................................A................................................... 194
    mRNA          1115 CTCCTCCGACGGCGGCGGCAGCAGGTGGACCTCGGGCCGGAATGTGTTTCCGGATTTTGAGATGTTGGCGGTTAGTGGGGTCTTGGACTC 1026
    PCR product   195  ..............................................................G.G..........C............. 284
    mRNA          1025 GGGGTGGGCAGCAGTGCAGGTGAA 1002
    PCR product   285  .....A............A..... 308

C   mRNA          661 GCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAGAGAGAGAAGTGCCCCCACCTGCTCCT 751
    PCR product   3   ...-....-................................................G........................... 91
    mRNA          752 CAGTTCCAGCCTGACCCCCTCCCATCCTTTGGCCTCTGACCCTTTTTCCACAGGGGACCTACCCCTATTGCGGTCCTCCAGCTCATCTTTCAC 846
    PCR product   92  ..................................................................................... 184
    mRNA          845 CTCACCCC 852
    PCR product   185 ........ 192

D   mRNA          407 CCACTGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTCCAAGCCAACAAGGCCACACTAGTGTGTCTGATCAGTGACTTCTACCCGGGAGCT 498
    PCR product   5   ....C.G..A......-......................T..................G...................... 95
    mRNA          499 GTGACAGTGGCCTGGAAGGCAGATGGCAGCCCCGTCAAGGCGGGAGTGGAGACCACCAAACCCTCCAAACAGAGCAACAACAAGTACGCG 590
    PCR product   96  ..................................................................................... 185
    mRNA          591 GCCAGCAGCTACCTGAGCCTGACGCCCGAGCAGTGGAAGTCCCACAGAAGCTACAGCTGCCAGGTCACGCATGAAGGGAGCACCGTGGAG 678
    PCR product   186 ....................................T.............................................. 275
    mRNA          679 AAGA 682
    PCR product   276 .... 279
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Figure 3. Alignment of the SqRT-PCR products sequences with the mRNA sequences from the NCBI database. (A-D) Alignment of the SqRT-PCR products sequences from HMCs with those of the Ig Cα1, Ig Cα2, Ig Cκ and Ig Cλ mRNA sequences from the NCBI database (Gene Bank, BC016369.1, BC073765.1, Y14736.1, X57823.1). SqRT-PCR, semi-quantitative reverse transcription-polymerase chain reaction; NCBI, National Center for Biotechnology Information; HMC, human mesangial cell.

of pro-inflammatory factors on the IgA expression in HMCs. 24 h after AngII stimulation, the immunofluorescence staining was stronger for Ig α, Ig κ, Ig λ in the cytoplasm (Fig. 6A). Ig α and Ig λ were more abundant on the fibrous structures in the cytoplasm and a granular accumulation was observed on the cell membranes. Similar to the WB results immunostaining revealed that Ig κ was expressed weakly in HMCs and its accumulation on the membranes was not obvious.

The association of IgA with cell apoptosis and cell adhesion. To investigate the possible effects of HMC-produced IgA on cell functions, we used the siRNA transfection method to down regulate the expression of Ig α1 in HMCs. After transfection with siRNAs for 48 h, the cells were collected to detect the Ig α1 expression in HMCs. The relative expression of the 53 kDa form of the Ig α1 was significantly downregulated in the siRNA-1 and the siRNA-3 treated groups compared to the negative control (NC) group (0.32 ± 0.01 vs. 0.73 ± 0.05, P<0.05; 0.36 ± 0.01 vs. 0.73 ± 0.05, P<0.05, respectively) and this was also similar with the expression of the 38 kDa form (0.34 ± 0.01 vs. 0.58 ± 0.03, P<0.05; 0.26 ± 0.02 vs. 0.58 ± 0.03, P<0.05) (Fig. 6B and C). These results indicated that siRNA-1 and siRNA-3 transfection could effectively down-regulate IgA in HMCs.

Annexin V assay combined with flow cytometry was performed to evaluate the apoptosis rates. After siRNA transfection for 48 h, early apoptosis in HMCs was detected. Apoptosis in the HMCs siRNA-1 and the siRNA-3 groups showed an early increase compared with that in the NC group (20.45 ± 5.34 vs. $10.27\pm5.31\%$, P<0.05; 27.25 ± 9.81 vs.

$10.27\pm5.31\%$, P<0.05, n=4) (Fig. 6D and E), indicating that IgA expression in HMCs might play an important role in cell growth and apoptosis. Cell adhesion ability is important for HMCs to execute functions such as structural support of the capillary tuft, modulation of glomerular hemodynamics and phagocytic removal of macromolecules and immune complexes. After siRNA transfection for 48 h, cell adhesion rates of HMCs in both the siRNA-1 and the siRNA-3 groups were significantly decreased compared to the NC group (34.99 ± 2.56 vs. $46.88\pm6.70\%$, P<0.05; 27.16 ± 4.67 vs. $46.88\pm6.70\%$, P<0.05) (Fig. 6F). The significant decrease of cell adhesion rates indicated that the knockdown of IgA in HMCs might inhibit cell adhesion. These changes in the rate of early apoptosis and the ability to adhere indicated that IgA expression is associated with mesangial cell functions.

Discussion

Definitive diagnosis of IgAN requires a kidney biopsy and IgAN is identified immunohistologically by the presence of dominant or co-dominant glomerular deposits of IgA (39), which had been generally considered to be B cell derived. The deposits consist predominantly of polymeric IgA structures of the IgA1 subclass (40). The pathogenic IgA deposition in the glomerular mesangium can activate mesangial cells and induce mesangial hyper-cellularity, apoptosis, oxidative stress, activation of complement, scarring in the glomerular and interstitial compartments, and secretion of pro-inflammatory factors, causing symptoms such as proteinuria, hematuria, and
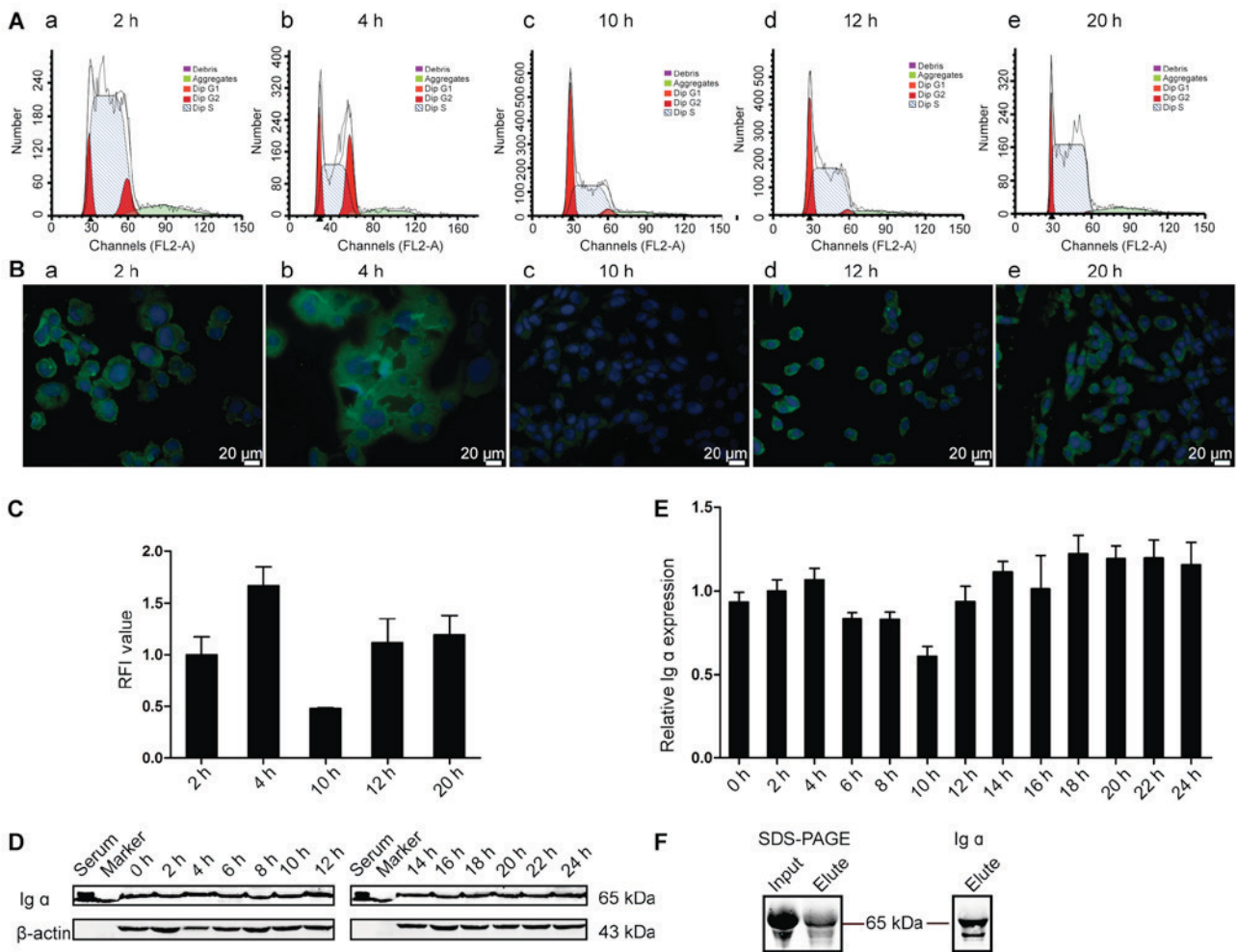
Figure 4. Dynamic detection of Ig α expression in HMCs and secretion. (A) The cell cycle analysis by flow cytometry from 2 to 24 h (a-e). (B and C) Ig α1 expression was measured as the RFI at different time points from 2 to 24 h by immunofluorescence staining. Green, antibody staining; blue, nuclear staining with DAPI; scale bar, 20 μm. (D and E) Expression of Ig α in the cell lysates at different time points after synchronization of HMCs. The data were standardized to the levels of β-actin. (F) The 65 kDa protein band of Ig α collected from the culture supernatant and purified by affinity chromatography with jacalin-sepharose; the band was further analyzed by mass spectrometry. HMC, human mesangial cell; RFI, relative fluorescence intensity; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Figure 5. Mass spectrometry results of the affinity purified 65 kDa protein band detected in the culture supernatant of the HMCs by immunostaining. Alignment of the amino acid sequence of the protein band from HMCs and those of Ig α1 (A) and Ig α2 (B) constant region sequences from the NCBI database. The black bolded letters indicated the matching amino acids from mass spectrometry results and NCBI database in the alignment. HMC, human mesangial cell; NCBI, National Center for Biotechnology Information.

leading to IgAN (41-43). Igs expression in non-B cells has been reported in recent years by several studies, which provided

clues for IgA expression in mesangial cells (11,14,37). Our study demonstrated, for the first time, that mesangial cells
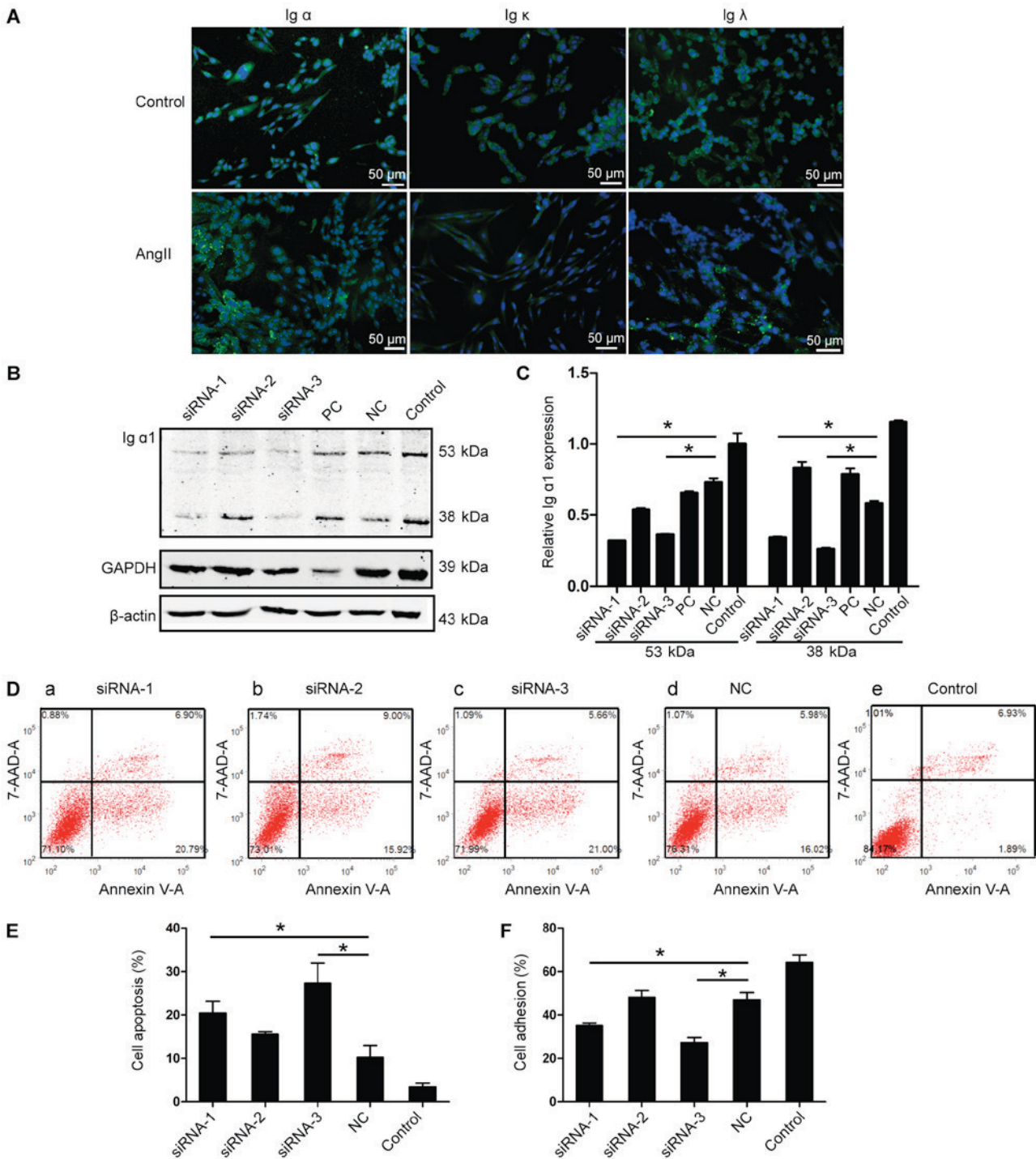
Figure 6. IgA expression after AngII stimulation and its effects on cell apoptosis and adhesion. (A) The positive expression of the Ig α, Ig κ and Ig λ proteins in HMCs, and after stimulation with AngII for 24 h, detected by immunofluorescence staining; scale bar, 50 µm; (B) Representative western blot images of Ig α1 knockdown in HMCs. (C) The expression rate of the 53 and the 38 kDa band of Ig α1 proteins; after Ig α1 siRNA-1 and siRNA-3 transfection for 48 h; transfection with siRNA-1 and siRNA-3 was repeated three times. The data were standardized to the levels of β-actin. (D and E) The rate of cell apoptosis after Ig α1 siRNA transfection for 48 h; n=4. (F) Cell adhesion of HMCs after siRNA transfection for 48 h; n=3. *P<0.05 vs. NC. siRNA-1, siRNA-2, siRNA-3: siRNA constructs targeting different regions of the Ig α1 heavy chain constant region; PC, positive control: siRNA targeted GAPDH; NC, negative control: Nonspecific, scrambled siRNA. HMC, human mesangial cell; siRNA, small interfering RNA.

may produce and secret IgA, and that the deposited IgA in the mesangium of patients with IgAN may be, at least partially, originated from mesangial cells.

In addition, in this study, we have demonstrated that the Ig α, Ig κ and Ig λ proteins are present in HRMCs and HMCs, and that their presence was not due to artificial contamination by B lymphocytes or by the FBS buffer in the culture

media. These results confirm that the IgA, especially IgA1, is expressed in the mesangial cells. The different molecular weights of the Ig α heavy chain suggested that Ig synthesis and assembly occur at different stages or that it existed in different truncated or aggregated forms, as it has been previously reported by Hu et al (44). Furthermore, our study has shown that the IgA heavy and light chain constant and

variable region gene transcripts and proteins were present in the HRMCs and HMCs and that their high homology with those mRNA sequences in the NCBI database strongly supports IgA expression in mesangial cells,. The unique or dominant Ig Vκ sequences in non-B cells are consistent with other reports (26,37).

Increase of early apoptosis and decrease of cell adhesion ability in HMCs after IgA downregulation were observed in our study, which indicated that expression of Ig α1 might be associated with mesangial cell functions such as apoptosis, proliferation, and adhesion. IgA mediated cell proliferation and apotosis has been reported in human epithelial cancer cells, but the mechanism was not investigated (45). Previous studies have shown that the pathogenesis of a variety of renal diseases is highly correlated with cell apoptosis and changes of apoptotic genes, in which the Bcl-2 family is one of the most implicated gene families (46,47). Besides, active effector caspases could proteolytically degrade a range of intracellular proteins during the apoptosis process (48,49). IgA expression may participate in the transcriptional and/or post-translational regulation of apoptosis related genes or proteins, such as caspases, to inhibit mesangial cell apoptosis. The specific molecules contributing to cell adhesion between the mesangial cell and the glomerular basement membrane are not clear. However, the protein called Epithelial Protein Lost In Neoplasm (EPLIN) was reported to strongly express in glomerular mesangial cells (50). EPLIN is implicated in the organization of the actin cytoskeleton, during the cell-cell or cell-matrix interactions (51). The above evidence provide us with clues to explore the underlying mechanism(s) regulating IgA expression in mesangial cells and mediating apoptosis and adhesion.

The results of our study have potential application and significance in clinical practice. First, the facts that IgA could be expressed in mesangial cell and secreted out of cell can illustrate that the IgA deposited in the mesangium in patients with IgAN may be, at least partially, originated from mesangial cells and may induce mesangial cells proliferation and secretion of extracellular matrixes. Second, our results have shown that mesangial cell-derived IgA is required for physiological cell functions, so exploring the factors which could lead to IgA deposition in the mesangium would be of great clinical significance. Third, the upregulation of Ig α, Ig κ, Ig λ expression by AngII in the cytoplasm of HMCs indicates that an interaction exists between angiotensin and IgA. This interaction can be potentially targeted for the clinical treatment of IgAN by exploiting angiotensin converting enzyme inhibitors or AngII receptor blockers.

Several points in the study need to be further clarified. First, IF staining demonstrated the presence of the κ chain in the cytoplasm and RT-PCR identified the transcript of the κ chain in the HMCs, but WB was not able to detect the Ig κ band. Generally speaking, antibodies used in IF recognize the three-dimensional structure while those in WB bind to the short line chain of the proteins, therefore the IF staining results are more convincing. The negative result in the WB might be attributed to the insufficient recognition by the antibodies. Secondly, a 65 kDa band after jacalin affinity chromatography was positively detected with antibodies against Ig α and Ig α1 but not Ig α2, however both α1 and α2 heavy chains were

detected in the band by mass spectrometry. IF demonstrated the staining of both α1 and α2 in the cytoplasm and RT-PCR showed that the transcripts of both α1 and α2 were expressed in HMCs. The dominant expression of Ig α1, as we found by IF and by WB, may compete with the binding of the antibody to the Ig α2. It was not easy to explain the affinity of jacalin to Ig α2, which was considered not to have any glycosylation sites at the hinge area, and it was unclear if the glycosylation at the hinge area of Ig α1 and Ig α2 in the mesangial cells was different from those in the plasm cell.

In conclusion, this study demonstrates that mesangial cells can express and secret IgA and that this expression may be associated with cell functions. Our findings provide clues for the implication of the HMC-produced IgA in the excessive deposition of IgA in the pathogenesis of IgAN.

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