Activation of tubular epithelial cells by mesangial-derived TNF-α: Glomerulotubular communication in IgA nephropathy

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Activation of tubular epithelial cells by mesangial-derived TNF-α: Glomerulotubular communication in IgA nephropathy.

Background. IgA nephropathy (IgAN), characterized by mesangial IgA deposition, runs a variable clinical course with tubulointerstitial damage and renal failure in no less than 30% of patients. Histologically, IgA is rarely detected in renal tubules. The direct toxicity by IgA on renal tubules remains uncertain. We hypothesize that mediators released from human mesangial cells (HMC) triggered by IgA deposition may lead to activation of proximal tubular epithelial cells (PTEC).

Methods. The binding of IgA to PTEC or HMC was assessed by flow cytometry. IgA-HMC medium was prepared by collecting the spent medium in which growth arrested HMC were incubated with IgA isolated from patients with IgAN, healthy control subjects, or other nephritic control patients. PTEC was cultured with the IgA-HMC medium in the presence or absence of neutralizing antibodies to TNF-α, IL-1β, TGF-β, or PDGF. Gene expression and protein synthesis of TNF-α, MIF, or ICAM-1 by PTEC were determined by RT-PCR and ELISA, respectively.

Results. The binding of IgA isolated from patients with IgAN to PTEC was increased when compared to binding of IgA from healthy control subjects ($P < 0.005$). However, the binding to PTEC was less than one tenth that of HMC in IgAN. The binding to PTEC was not mediated through known IgA receptors, as shown by competitive binding assays and gene expression of the receptors. Despite the in vitro binding, PTEC cultured with isolated IgA exhibited no increased cell proliferation or enhanced synthesis of TNF-α, MIF, or sICAM-1. However, when PTEC were cultured with IgA-HMC medium prepared from IgAN patients, there was enhanced proliferation of PTEC ($P < 0.001$) and increased synthesis of TNF-α, MIF, and sICAM-1 when compared with PTEC cultured with IgA-HMC medium from control subjects ($P < 0.001$). The synthesis of MIF and sICAM-1 by PTEC cultured with IgA-HMC medium was reduced by neutralizing antibodies to TNF-α ($P < 0.001$) but not by neutralizing antibodies to IL-1β, TGF-β, or PDGF.

Conclusion. Our finding implicates that TNF-α released from the mesangium after IgA deposition activates renal tubular cells. The glomerulotubular communication could play an important role in the pathogenesis of tubulointerstitial damage in IgAN.

IgA nephropathy (IgAN), the most common form of glomerulonephritis, is one of the leading causes of renal failure in many parts of the world. The disease is characterized by mesangial deposition of pathogenic polymeric IgA1 (pIgA1) [1], proliferation of mesangial cells, increased synthesis of extracellular matrix (ECM), and infiltration of macrophage, monocytes, and T cells [2, 3]. IgAN runs a highly variable clinical course with a slowly relentless and progressive renal failure in 30% to 50% of patients within 30 years after first clinical presentation [4]. A subgroup of IgAN with severe tubulointerstitial atrophy is often associated with the most rapid progression to end-stage renal failure [5]. The severity of tubulointerstitial damage in different glomerulonephritides, including IgAN, correlates closely with the declining renal function and the long-term clinical outcome [6–9]. Inflammation elicited by infiltrating mononuclear cells plays an important role in the development of glomerular and tubulointerstitial injury in IgAN [10–13]. Proteinuria is a potent inflammatory stimulus of proximal tubular epithelial cells (PTEC). Our recent work has provided experimental evidence to suggest a tubuloglomerular "cross-talk" mechanism, involving different soluble factors, and is likely to operate in different glomerular and interstitial nephritis [14]. However, high-grade or nephrotic-range proteinuria is not a common clinical feature of IgAN. Furthermore, IgA deposits are rarely detected in the tubulointerstitium [15, 16]. Hence, the most important and central question is how IgA deposits in glomerular mesangial cells lead to subsequent tubulointerstitial damage resulting in end-stage renal failure. Normally, the glomerular barrier is impermeable to proteins. However, increase in glomerular barrier pore size that allowed the passage of proteins to the tubular lumen is observed in various

glomerular diseases [17]. Hence, the tubulointerstitial damage in IgAN may be the result of cross-talk or communication between the glomerular and the tubular compartment due to increased passage of mediators through the glomerular barrier. Mediators released by infiltrating cells or resident kidney cells (including mesangial cells and PTEC) are directly responsible for activation of PTEC. The inflammatory cascade may be amplified by local production of chemokines, cytokines, adhesion molecules, and extracellular matrix components, which ultimately leads to progressive loss of kidney function [18–20]. So far, the interaction or cross-talk between these individual components that may lead to tubulointerstitial injury in IgAN has not been addressed. In this study, we first explored the expression of IgA receptors and examined any direct effect of IgA molecules on PTEC. The pathophysiologic effects on PTEC by mediators released from mesangial cells triggered by IgA deposition were also studied.

METHODS

Materials

Jacalin agarose was purchased from Pierce (Rockford, IL, USA). Rosewell Park Memorial Institute Medium (RPMI 1640 medium), Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12 medium) and fetal bovine serum (FBS) were obtained from Life Technologies (Rockville, MD, USA). F(ab')$_2$ fragment of FITC-conjugated rabbit antihuman IgA and FITC-conjugated preimmune rabbit Ig antibodies were obtained from Dako (Kyoto, Japan). 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) was obtained from Molecular Probe (Eugene, OR, USA). Anti-secretory component (Anti-sc) was obtained from ICN Pharmaceuticals (Aurora, OH, USA). Anti-CD89 blocking antibody (Clone My43) was obtained from Medarex (Annandale, NJ, USA). Total IgA was prepared by affinity chromatography with jacalin agarose. Polymeric IgA (pIgA) and monomeric IgA (mIgA) were further separated by size exclusion chromatography using fast protein liquid chromatography as previously described [21]. The endotoxin content in the IgA preparations and the spent medium were determined using *Limulus* amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD, USA). The endotoxin level for all IgA preparation was <1 pg/mg protein. Asialo-orosomucoid (ASOR) was prepared by desialylation of human orosomucoid with neuraminidase (0.03 U/mg protein) following incubation for 8 hours at 37°C in 0.1 mol/L sodium acetate buffer, pH 5.0 [21]. Neutralizing antibodies to tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), or platelet-derived growth factor (PDGF) were obtained from R&D Systems (Min-

neapolis, MN, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Patients and control subjects

Twenty-two Chinese patients (10 male and 12 female) with clinical and renal immunopathologic diagnosis of primary IgAN were studied. IgA nephropathy was diagnosed by the presence of predominant granular IgA deposits, mainly in the glomerular mesangium and occasionally along the peripheral capillary basement membrane by immunofluorescene examination, and the presence of mesangial electron-dense deposits in ultrastructural examination. All the patients were symptomatic for more than 12 months and no significant renal impairment was documented. Systemic lupus erythematosus, Henoch-Schönlein purpura (HSP), and hepatic diseases were excluded by detailed clinical history, examination and negative laboratory findings for hypocomplementemia, anti-DNA antibody, or hepatitis B virus surface antigen. Twenty milliliters of blood was collected from each patient at clinical quiescence. The serum was isolated and frozen at −20°C until isolation of IgA by jacalin-agarose affinity column.

Fifteen healthy subjects (7 male and 8 female), comparable in age and race, with no microscopic hematuria or proteinuria, were recruited as normal control subjects. Another 15 patients, 10 with minimal change nephropathy (MCNS) and 5 with HSP, were also recruited as disease control patients. Serum was similarly collected from these individuals for processing. Informed written consent for blood sampling was obtained from every subject. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the university and hospital ethics committees.

Cell culture

Cell lines, including the colonic adenocarcinoma line (HT29), the hepatocellular line (HepG2), and the monocytic line (U937), were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). These cell lines, cultured under the condition recommended by ATCC, were used as positive or negative control in various experiments. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll gradient separation. Isolation and characterization of human mesangial cells (HMC) were performed as previously described [22]. Glomerular cells were grown in RPMI 1640 medium supplemented with glutamine (2 mmol/L), *N*-[2-hydroxyethyl]-piperazine-*N*'-[2-ethanesulphonic acid] (HEPES) (10 mmol/L), penicillin (50 U/mL), streptomycin (50 μg/mL) and 12% FBS. The cells were incubated at 37°C in 5% CO_2 and 95% air. Mesangial cells have a stellate appearance and grow in clumps. They show a network of intracellular fibrils of

Table 1. Primer sequences and size of PCR products

PCR product	5′ to 3′ sequences of PCR primers	Size bp
FcαR	5′-TCG GGG TCT GGT GTT GAT GC-3′ 5′-GGA AAG TGT GGT GGG GAA GC-3′	415
ASGPR H1	5′-AGC AAC TTC ACA GCG AGC AC-3′ 5′-TCC CGT CCA CCC ACT TCC AG-3′	448
ASGPR H2	5′-GCA GGC TGA ATC CCA GGA GA-3′ 5′-CCA TTG AAG AGG CTG ACG AT-3′	1072
pIgR	5′-GCA GTG GGG AAA ACT GTG AC-3′ 5′-GTC GCG GGG AAT CGT GGA CT-3′	245
Fcα/μR	5′-GAC AAC TAC CAA GGC TGA TAG G-3′ 5′-TCT GTC CCT CAG GGT CCT GGA T-3′	702
TfR	5′-TTC TTT CTG TTT TTG CGA GGA C-3′ 5′-AAC GTC ACC AGA GAG GGC ATT T-3′	601
TNF-α	5′-CAA TGG CGT GGA GCT GAG AGA-3′ 5′-CCA AAG TAG ACC TGC CCA GAC-3′	345
ICAM-1	5′-GAG ACC CCG TTG CCT AAA-3′ 5′-CCG CAG GTC CAG TTC AGT-3′	399
MIF	5′-CTC TCC GAG CTC ACC CAG CAG-3′ 5′-CGC GTT CAT GTC GTA ATA GTT-3′	255
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′ 5′-TCC ACC ACC CTG TTG CTG TA-3′	452

myosin and contract in the presence of 1 nmol/L of angiotensin II. Human PTEC were isolated as previously described [23]. Tubular cells were grown in DMEM/F12 medium supplemented with glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), hydrocortisone (40 ng/mL), and 10% FBS at 37°C in 5% CO_2 and 95% air. These cells were characterized to be of proximal tubular origin by immunofluorescence and enzyme histochemistry [14]. In all experiments, cultured cells grown to confluence were growth arrested with culture medium containing 0.5% FBS for 48 hours before the commencement of experiments.

Total RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR)

Total cellular RNA was extracted using NucleoSpin RNA II total RNA extraction kit (Macherey-Nagel, Duren, Germany). The quality of the extracted RNA was monitored by formaldehyde agarose gel electrophoresis. RNA was quantified by absorbance at 260 nm. Four micrograms of total RNA was reverse transcribed to cDNA with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) in a 20 μL reaction mixture containing 50 ng random hexamer, 0.5 mmol/L dNTPs, and 20 U of RNase inhibitor. The cDNA was stored at −20°C until further use. Gene expression of Fc alpha receptor 1 (FcαR or CD89), H1 and H2 chains of asialoglycoprotein receptor (ASGPR H1 and ASGPR H2), polymeric-immunoglobulin receptor (pIgR), Fc alpha/mu chain receptor (Fcα/μR), transferrin receptor (TfR or CD71), TNF-α, macrophage migration inhibitory factor (MIF), and intercellular adhesion molecule-1 (ICAM-1) by PTEC were examined by PCR using specific primers designed from known sequences in the GenBank. The primer sequences are depicted in Table 1. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Images of the gel were captured using the Gel Doc 1000 gel Documentation System from Bio-Rad (Hercules, CA, USA). Gene expression of TNF-α, MIF, and ICAM-1 was then analyzed by the IP Lab gel software from Signal Analytics (Vienna, VA, USA), and the results were expressed

as a ratio of the target amplicon to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) amplicon. Full precaution was taken for all necessary steps to ensure the validity of the results as previously described [24].

Flow cytometry

Human mesangial cells or PTEC were grown to log phase and harvested by using 0.05% trypsin/0.02% EDTA for 5 minutes at room temperature. The cells were adjusted to 5×10^6 per mL, and 200 μL of cell suspension were used in binding assays. Staining was performed at 4°C with staining buffer consisting of phosphate-buffered saline (PBS) with 1% FBS and 0.1% sodium azide. The cells were incubated with 100 μL of IgA (final concentration 50 μg/mL) for 30 minutes. After incubation, the cells were washed with staining buffer and then further incubated with 100 μL of rabbit antihuman IgA antibody. Background control staining was achieved by reaction with preimmune F(ab')$_2$ fragment of fluorescein conjugated isotypic antibody. The stained cells were analyzed using a Coulter EPICS XL analyzer (Miami, FL, USA). A minimum of 5000 fixed cells for each sample was analyzed. Fluorescence intensity was evaluated by comparing the mean fluorescence channels. The result was expressed as mean fluorescence intensity (MFI).

Inhibition study

The fluorescein-conjugated pIgA (FITC-pIgA) or mIgA (FITC-mIgA) was prepared from IgA preparation from each individual patient with IgAN using the FluoReporter Protein Labeling Kits (Molecular Probe). The effect of different potential blocking proteins on IgA binding was examined by flow cytometry. Proximal tubular epithelial cells (1×10^6 cells in staining buffer) were incubated with different blocking proteins, including: 5 mg/mL unlabeled pIgA, 5 mg/mL unlabeled mIgA, 5 mg/mL unlabeled IgG, 5 mg/mL unlabeled IgM, 50 μg/mL Anti-sc, 5 mg/mL ASOR, 5 mg/mL orosomucoid, 50 μg/mL My43, and 5 mg/mL transferrin, for 30 minutes at 4°C. After incubation, the cells were washed once with staining buffer before incubation with

0.5 mg/mL FITC-mIgA or FITC-pIgA. Following incubation, the stained cells were washed and analyzed by flow cytometry as described above.

Preparation of spent (conditioned) medium

Growth-arrested HMC were cultured in 6-well culture plates (1×10^6 cells per well) with culture medium containing 0.5% FBS and IgA (final concentration 50 μg/mL) isolated from patients with IgAN, healthy control subjects, and disease control patients for 48 hours. The spent medium (IgA-HMC medium) after culture was collected and kept frozen at $-70°C$ until used. Spent medium from HMC cultured without the addition of IgA preparation (control medium) was used as control. For examining the effect of IgA-HMC medium on PTEC, different preparations of IgA-HMC medium were diluted 10-fold with DMEM/F12 containing 0.5% FBS for culturing PTEC. Growth-arrested PTEC were seeded onto 6-well culture plates (1×10^6 cells per well) and were cultured with diluted IgA-HMC medium for either 24 (for RT-PCR) or 48 hours (for ELISA). After culture, the cells were collected for total RNA isolation, and the supernatant was stored at $-70°C$ before assay of various cytokines and chemokines. In order to study whether cytokine and chemokine synthesis by PTEC was induced via TNF-α in the IgA-HMC spent medium, similar experiments were performed in cells incubated with antibodies against TNF-α (0.1 μg/mL), IL-1β (0.1 μg/mL), TGF-β (10 μg/mL), or PDGF (100 μg/mL) 1 hour before stimulating with different spent medium. The concentration of neutralizing antibodies used was determined by a preliminary experiment that demonstrated that TNF-α (up to 1 ng/mL) induced up-regulation of MIF synthesis in PTEC was abolished by incubating cells with neutralizing anti-TNF-α at 0.1 μg/mL. The concentrations of various neutralizing antibodies used were determined by preliminary dose-determining experiments. MIF synthesis up-regulated by TNF-α (up to 1 ng/mL), cell proliferation induced by PDGF (up to 10 ng/mL), IL-6 synthesis induced by IL-1β (up to 10 ng/mL), or fibronectin synthesis induced by TGF-β (up to 10 ng/mL) in PTEC was abolished by incubating cells with neutralizing anti-TNF-α at 0.1 μg/mL, anti-PDGF at 100 μg/mL, anti-IL-1β at 0.1 μg/mL, or anti-TGF-β at 10 μg/mL, respectively.

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Proliferation of cultured PTEC was determined using a MTT assay kit (R&D Systems). Briefly, growth-arrested PTEC were seeded onto 96-well plates (1×10^5 cells per well) before stimulated with IgA preparation or IgA-HMC medium for 48 hours. MTT reagent was then added and incubated at $37°C$ until purple precipitate was clearly visible under an inverted microscope. Detergent reagent
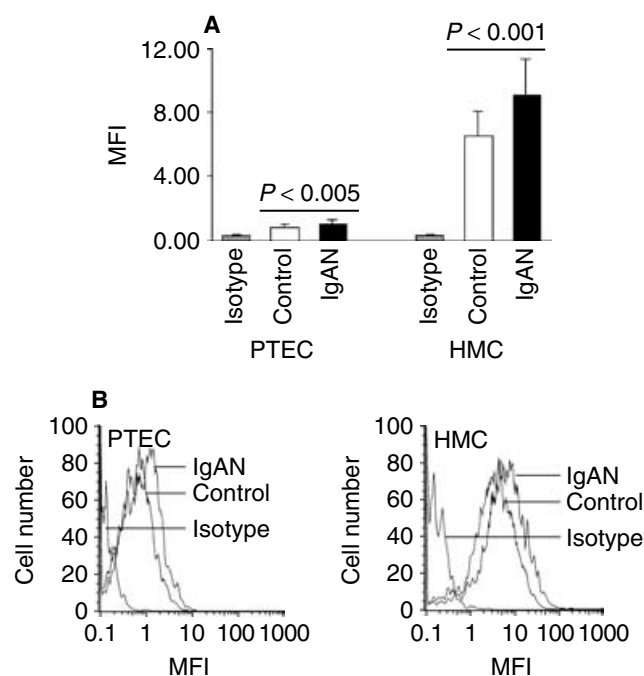

Fig. 1. Flow cytometry assay of IgA binding to PTEC and HMC. (*A*) Binding of IgA from IgAN patients ($N = 22$) to PTEC and HMC is increased when compared with healthy control subjects ($N = 15$). (*B*) Representative histogram showing a lower binding of IgA to PTEC than to HMC when same amount of IgA (50 μg/mL) and the equal number of cells (1×10^6 cells) are used for the experiments.

was finally added and incubated in dark at $37°C$ until the purple precipitate was solubilized. The absorbance was measured using 550 nm as the primary wavelength and 650 nm as the reference wavelength.

ELISA of TNF-α, MIF, sICAM-1, IL-1β, TGF-β, and PDGF

Human TNF-α and soluble intercellular adhesion molecule-1 (sICAM-1) were measured by commercially available ELISA systems (Bender MedSystems, Vienna, Austria). Human IL-1β, TGF-β, and PDGF-B were assayed using sandwich ELISA (R&D Systems). Immunoreactive MIF was quantitated by a sandwich ELISA as described previously [25].

Statistical analysis

All data were expressed as mean ± standard deviation (SD) unless otherwise specified. Statistical difference was analyzed with multivariate analysis of variance (ANOVA) for repeated measures. All *P* values quoted are two-tailed, and the significance is defined as $P < 0.05$.

RESULTS

Binding of IgA to PTEC and HMC

Figure 1A shows the binding of total IgA to PTEC or HMC determined by flow cytometry. The binding of
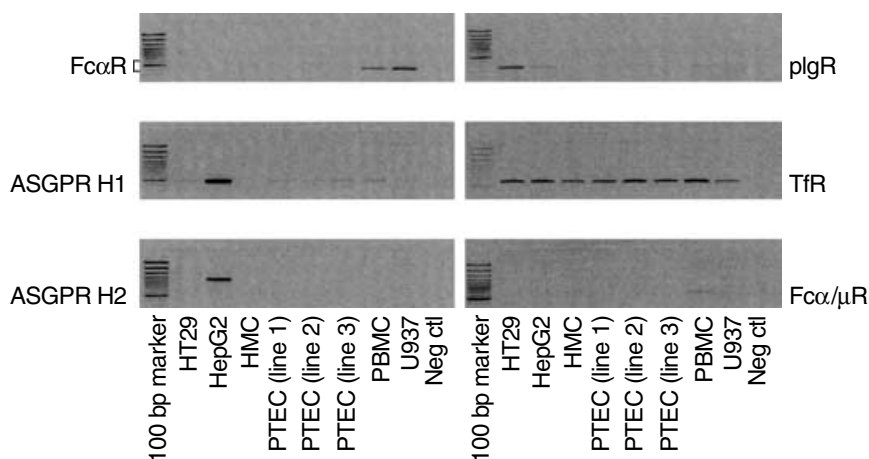
Fig. 2. mRNA expression of IgA receptors by reverse transcription-polymerase chain reaction (RT-PCR). PTEC do not express mRNA of known IgA receptors, including FcαR, pIgR, and Fcα/μR, except TfR. As for ASGPR, only the H1 chain of the ASGPR is expressed in PTEC.

Table 2. Semiquantitative analysis of the expression of IgA receptors (from zero to 3+)

	FcαR	ASGPR H1	ASGPR H2	pIgR	TfR	Fcα/μR	GAPDH
HT29	–	+	–	++	+++	–	+++
HepG2	–	++++	+++	+	+++	–	+++
HMC	–	–	–	–	+++	+	+++
PTEC-1	–	+	–	–	+++	–	+++
PTEC-2	–	+	–	–	+++	–	+++
PTEC-3	–	+	–	–	+++	–	+++
PBMC	++	++	–	Weakly +	+++	+	+++
U937	+++	Weakly +	–	–	+++	–	+++
Negative control	–	–	–	–	–	–	–

IgA from IgAN patients to either cell type was higher than the binding of IgA from healthy control subjects (PTEC: 1.02 ± 0.26 vs. 0.82 ± 0.52; $P < 0.005$, and HMC: 9.07 ± 2.30 vs. 6.52 ± 1.55; $P < 0.001$). However, using identical concentration of IgA and same number of cells in the binding assay, the binding of IgA to PTEC was only 8.6% and 8.9% of the binding of IgA to HMC for IgAN patients and healthy control subjects, respectively (Fig. 1B).

Expression of surface IgA receptors in PTEC

Figure 2 depicts the mRNA expression of the FcαR, H1 and H2 chains of ASGPR, pIgR, Fcα/μR, and the transferrin receptors (TfR) in PTEC. Human mesangial cells and other cell lines were used as positive control for gene expression of different receptors. The findings are summarized in Table 2. PTEC did not express mRNA for any known IgA receptors except the transferrin receptor. The expression pattern was identical when three individual PTEC lines from different donors were used. Although the H1 chain mRNA was expressed in PTEC, there was no expression of ASGPR H2 chain, which was required for assembling a functional ASGPR.

Inhibition study of IgA binding to PTEC by competitor proteins

Figure 3 shows the inhibition studies in IgA binding to PTEC. The binding of FITC-mIgA or FITC-pIgA to PTEC was not blocked by preincubation with proteins that competitively blocked individual known IgA receptors, including IgM, anti-sc, ASOR, orosomucoid, anti-FcαR1 (clone My43), and transferrin. The binding of FITC-mIgA was reduced by 75% and 87% when the cells were preincubated with unlabeled mIgA and pIgA, respectively ($P < 0.0001$ for both). The binding of FITC-pIgA to PTEC was only reduced by 42% following preincubation with unlabeled mIgA ($P < 0.005$). In contrast, the binding of FITC-pIgA to PTEC was markedly reduced (~90%) following preincubation with unlabeled pIgA ($P < 0.0001$).

Cell proliferation of PTEC cultured with IgA

Despite the detection of binding of mIgA or pIgA to cultured PTEC, enhanced cell proliferation (measured by MTT assay) was not observed when incubated with IgA isolated from patients with IgAN or healthy control subjects (data not shown). The lack of cell proliferation was supported by the findings that the synthesis of TNF-α, MIF, or sICAM-1 in PTEC did not differ between culture experiments with IgA preparations from IgAN patients, healthy control subjects, or disease control patients (Fig. 4). The findings in gene expression of these mediators paralleled those of protein synthesis (data not shown).
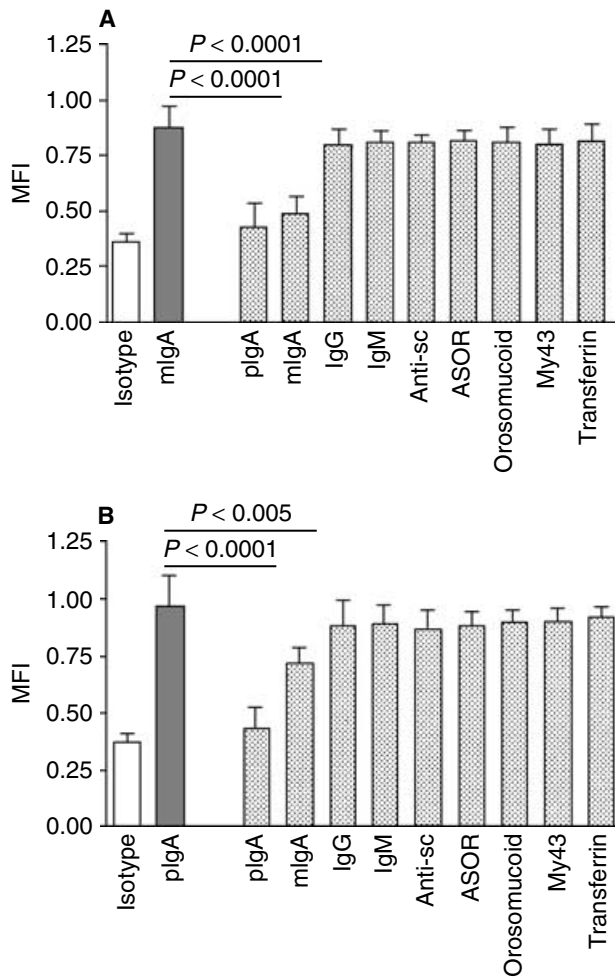
Fig. 3. Inhibition binding assay. Inhibition of competitor proteins on the binding of (*A*) FITC-mIgA or (*B*) FITC-pIgA to PTEC. The binding of FITC-mIgA or FITC-pIgA (solid bars) to PTEC is not blocked by preincubating PTEC with different competitor proteins (dotted bars). Open bar represents the background MFI using isotype antibody as control. Preincubation of PTEC with mIgA and pIgA results in reduction of FITC-mIgA binding by 75% and 86.5%, respectively. Preincubation of PTEC with mIgA and pIgA results in reduction of FITC-pIgA binding by 42% and 90%, respectively. The results represent the mean ± standard deviation from 5 individual experiments.

Cell proliferation study in PTEC cultured with IgA-HMC medium

We then explored whether IgA-HMC medium from patients with IgAN exhibited any stimulatory effect on cultured PTEC. Cell proliferation was higher in PTEC incubated with IgA-HMC medium from patients with IgAN than that from healthy control subjects ($P < 0.001$) (Fig. 5). However, IgA-HMC from other disease control patients (MCNS and HSP) did not exert any enhancement on the proliferation of PTEC.

Gene expression and protein synthesis of TNF-α, MIF, and ICAM-1 by PTEC cultured with IgA-HMC medium

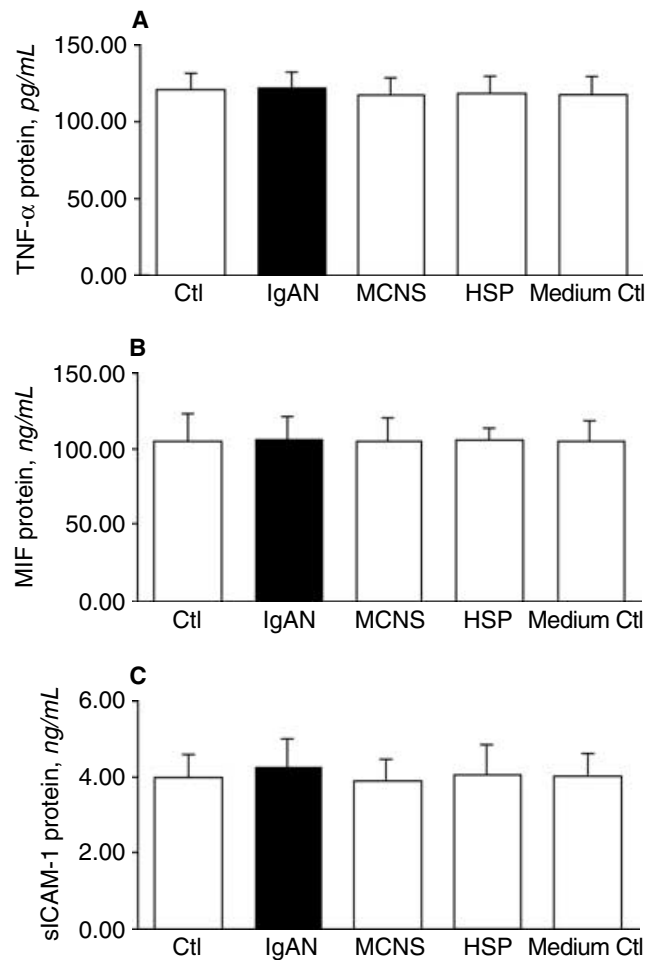Both gene expression and protein synthesis of TNF-α, MIF, and ICAM-1 were up-regulated when PTEC were

Fig. 4. Protein synthesis of TNF-α, MIF, and sICAM-1 in PTEC incubated with IgA. There is no difference in protein synthesis of (*A*) TNF-α, (*B*) MIF, and (*C*) sICAM1 when PTEC are incubated with total IgA prepared from patients with IgA nephropathy (IgAN), minimal change nephropathy (MCNS), or Henoch-Schönlein purpura (HSP) as compared with IgA from healthy control subjects (Ctl) or incubation with culture medium alone (Medium Ctl).
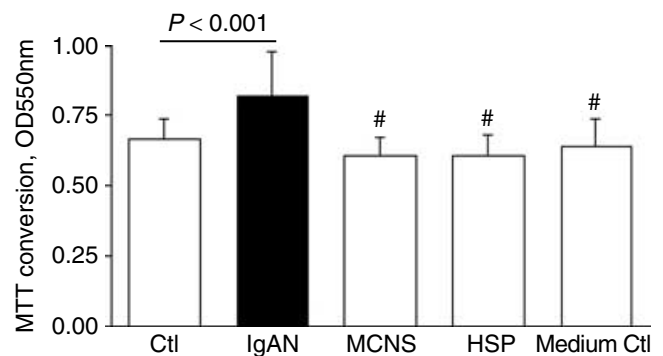

Fig. 5. Cell proliferation assay of PTEC incubated with IgA-HMC spent medium. Increased proliferation of PTEC is observed when cultured with IgA-HMC spent medium prepared from IgAN patients when compared with healthy control subjects (Ctl). PTEC incubated with IgA-HMC spent medium from patients with minimal change nephropathy (MCNS) or with Henoch-Schönlein purpura (HSP) demonstrate no increased proliferation when compared to IgA isolated from healthy control subjects. #$P < 0.001$ vs. IgAN.
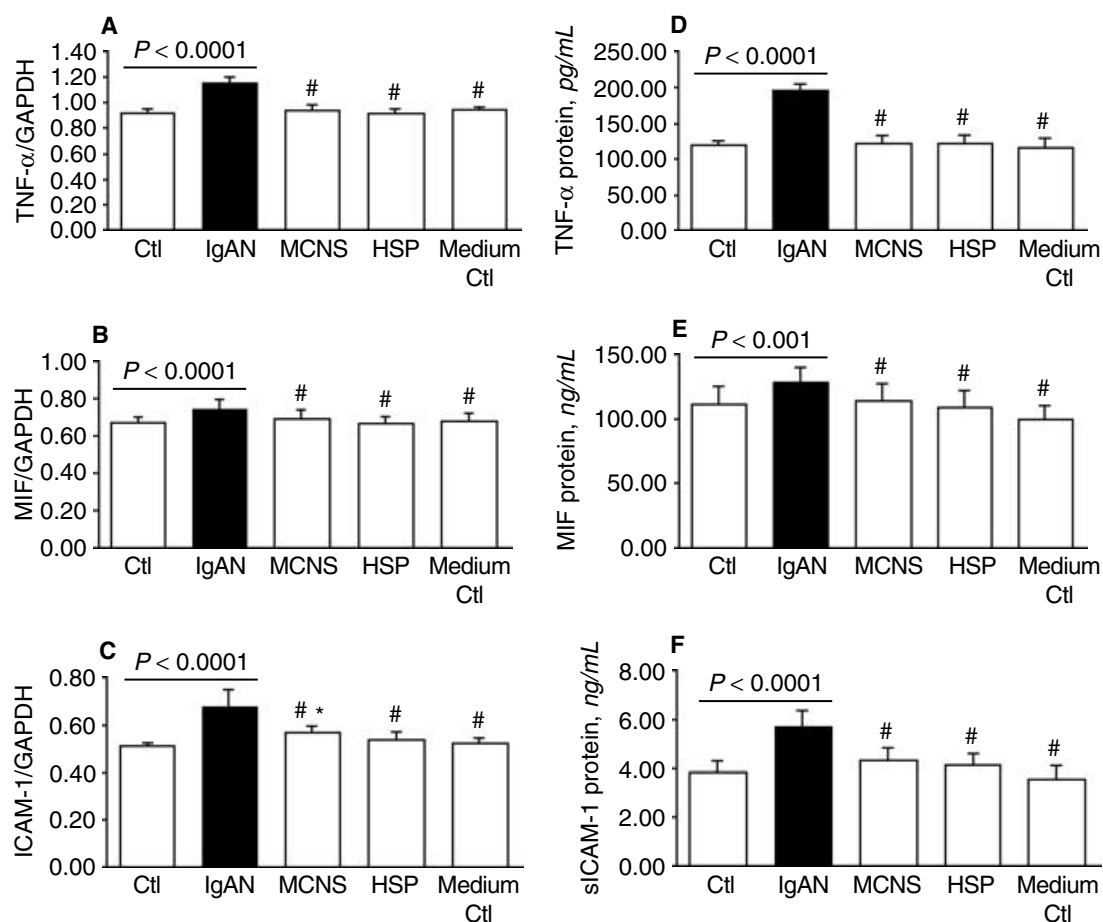
Fig. 6. Expression of TNF-α, MIF, and ICAM-1 in PTEC incubated with IgA-HMC spent medium. The gene expression of (*A*) TNF-α, (*B*) MIF, and (*C*) ICAM-1 is up-regulated in PTEC incubated with IgA-HMC spent medium prepared from IgAN patients when compared to that from healthy control subjects (Ctl). Gene expression of TNF-α or MIF is not increased in PTEC incubated with IgA-HMC spent medium prepared from patients with minimal change nephropathy (MCNS) or with Henoch-Schönlein purpura (HSP) when compared with that of healthy control subjects. The protein synthesis of (*D*) TNF-α, (*E*) MIF, and (*F*) sICAM-1 is up-regulated in PTEC incubated with IgA-HMC spent medium prepared from IgAN patients when compared to that from healthy control subjects (Ctl). Protein synthesis of TNF-α, MIF, or sICAM-1 is not increased in PTEC incubated with IgA-HMC spent medium prepared from patients with minimal change nephropathy (MCNS) or with Henoch-Schönlein purpura (HSP) when compared with that of healthy control subjects. #$P < 0.01$ vs. IgAN and *$P < 0.01$ vs. Ctl.

incubated with IgA-HMC medium from IgAN patients when compared with IgA-HMC from healthy control subjects ($P < 0.001$) or from disease control patients ($P < 0.01$) (Fig. 6). There was no increase in gene expression or protein synthesis of TNF-α and MIF in PTEC incubated with IgA-HMC medium from patients with MCNS or HSP when compared with that of healthy control subjects. However, the gene expression of ICAM-1 was slightly increased in PTEC incubated with IgA-HMC medium from patients with MCNS when compared with that of healthy control subjects ($P < 0.01$).

The concentration of TNF-α, IL-1β, TGF-β, and PDGF in the IgA-HMC medium

The concentration of TNF-α in the IgA-HMC medium was determined to rule out the possibility that the level of TNF-α synthesis by PTEC after cultured with IgA-

HMC medium was directly derived from the IgA-HMC medium. The synthesis of TNF-α was up-regulated in HMC incubated with IgA from patients with IgAN (Fig. 7). Following a 10-fold dilution, the calculated TNF-α concentration of the diluted spent medium used in culture experiments was 43.27 pg/mL. This concentration was less than one quarter of the supernatant concentration of PTEC cultured with the diluted spent medium from HMC incubated with IgA from IgAN patients. The synthesis of TGF-β was up-regulated in HMC incubated with IgA from patients with IgAN when compared with healthy control subjects (397.16 ± 49.60 pg/mL vs. 308.89 ± 39.02 pg/mL; $P = 0.0003$). There was no induction of PDGF synthesis in HMC incubated with IgA from patients with IgAN or control subjects as compared with basal PDGF level (data not shown). No IL-1β was detected in the medium of HMC incubated with IgA from patients or control subjects.
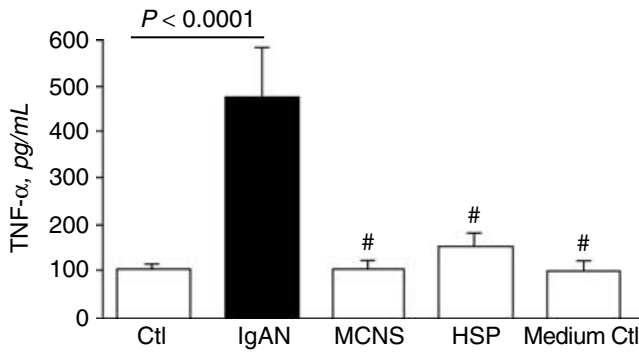
Fig. 7. Synthesis of TNF-α in HMC incubated with IgA. TNF-α is up-regulated when HMC are incubated with IgA prepared from IgAN patients as compared to healthy control subjects (Ctl). TNF-α synthesis is not increased in HMC incubated with IgA prepared from patients with minimal change nephropathy (MCNS) or with Henoch-Schönlein purpura (HSP) when compared with that of the Ctl. #$P < 0.001$.
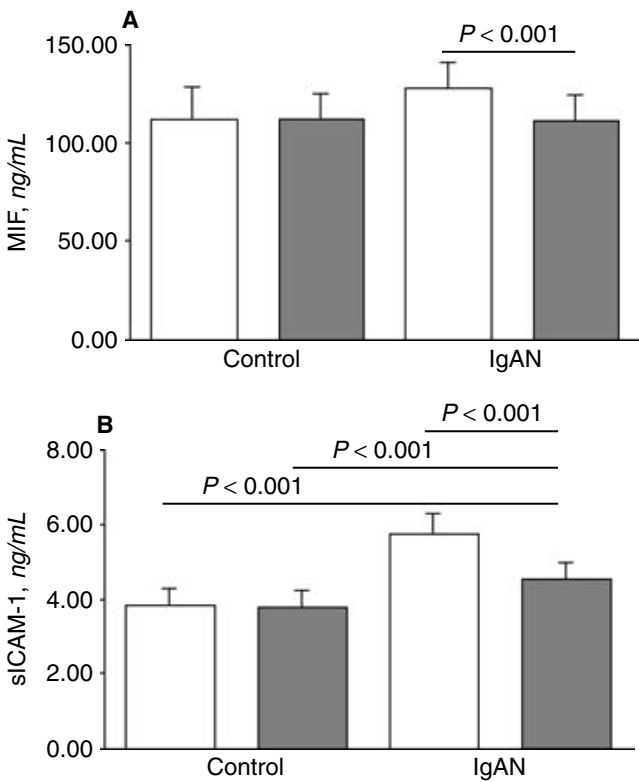

Fig. 8. Synthesis of MIF and sICAM-1 by PTEC incubated with IgA-HMC spent medium in the presence of a neutralizing anti-TNF-α antibody. A neutralizing anti-TNF-α at a concentration of 0.1 μg/mL (shaded bar) down-regulates (*A*) MIF and (*B*) sICAM-1 synthesis in PTEC incubated with IgA-HMC spent medium from patients with IgAN as compared with IgA-HMC spent medium from control subjects.

Effect of neutralizing antibodies in the protein synthesis of MIF and sICAM-1 by PTEC cultured with IgA-HMC medium

We then investigated the potential effect of a neutralizing anti-TNF-α antibody in modulating the protein
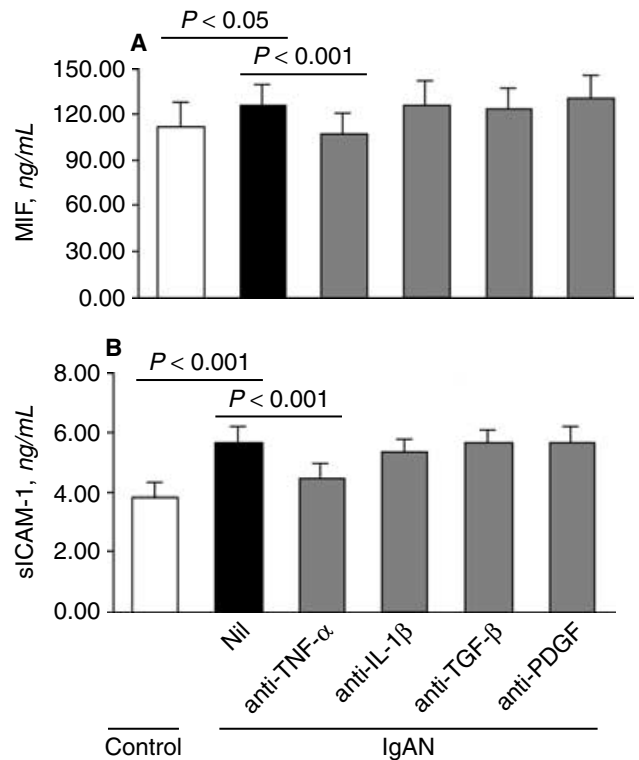

Fig. 9. Synthesis of MIF and sICAM-1 by PTEC incubated with IgA-HMC spent medium in the presence of neutralizing antibodies to TNF-α, IL-1β, TGF-β, or PDGF. Only the neutralizing anti-TNF-α (shaded bar) down-regulates (*A*) MIF and (*B*) sICAM-1 synthesis in PTEC incubated with IgA-HMC spent medium (black bar) from patients with IgAN as compared with IgA-HMC spent medium from control subjects (white bar). Synthesis of MIF or sICAM-1 is not reduced by neutralizing antibodies to IL-1β, TGF-β, or PDGF.

synthesis of MIF and sICAM-1 in PTEC incubated with IgA-HMC medium from patients with IgAN. Anti-TNF-α antibody at a concentration of 0.1 μg/mL completely abolished MIF protein synthesis (when compared with IgA-HMC medium from control) (Fig. 8A) and down-regulated sICAM-1 synthesis by 39% (Fig. 8B). Of note, the down-regulatory effect on MIF (Fig. 9A) or sICAM-1 synthesis (Fig. 9B) was only demonstrated in neutralizing anti-TNF-α antibody, but not with neutralizing antibodies to IL-1β, TGF-β, or PDGF.

DISCUSSION

In IgAN, mesangial IgA deposition is an early event that may subsequently lead to glomerular and tubular damages in a slowly but progressive clinical course. The binding of IgA to HMC is increased in IgAN [1, 26]. The interaction of IgA with glomerular mesangial cells induces cell proliferation [27] and the release of cytokines and chemokines [26, 28]. Tubulointerstitial damage leading to end-stage renal failure is a common sequel in most chronic kidney disease, including IgAN [9]. Increase in

urinary IgA concentration has been reported in IgAN, and the level of urinary IgA correlates with the serum creatinine as well as the urinary protein excretion [28]. In addition, the duration and extent of proteinuria are useful predictors for glomerular and interstitial damage in IgAN [29]. It is possible that the tubular lumen is constantly exposed to the high molecular weight IgA from patients with IgAN, especially when glomerular size barrier is impaired in IgAN [30]. Because significant proteinuria or nephrotic syndrome is not a common finding in IgAN, it is, therefore, important to know whether IgA can bind to PTEC and elicit any functional activation as observed in HMC.

We found increased binding of IgA from patients with IgAN to cultured PTEC as compared to binding of IgA from healthy control subjects. However, the quantity of IgA bound to PTEC was less than one tenth that of HMC in IgAN, thus raising the question whether the binding observed in the cell culture experiments was not specific. We then examined the expression of known IgA receptors to determine the binding was mediated through these receptors. We demonstrated that PTEC did not express any known IgA receptors except for the TfR and the H1 chain of the ASGPR, which failed to assemble a functional ASGPR due to the lack of the H2 chain. To further exclude whether the binding of IgA to PTEC was through the TfR or the other known IgA receptors, we conducted competitive binding studies using different ligands for known IgA receptors. Different competitor proteins for IgA receptors did not inhibit the binding of FITC-labeled pIgA or mIgA to PTEC. Most interestingly, we failed to show blocking of the IgA binding to PTEC by transferrin. Our observation is in accordance with the recent report showing that glomerular IgA deposition was only associated with the expression of glomerular TfR, but not with the tubular TfR [31]. Furthermore, despite positive immunostaining of TfR that was evident in glomerular mesangium and renal tubules, enhanced expression of TfR was only observed in the mesangium in patients with IgAN. The lack of cell proliferation and absence of functional activities measured by synthesis of TNF-α, MIF, or sICAM-1 in PTEC incubated with IgA preparations from IgAN adds further support to the notion that the low level of IgA binding to cultured PTEC represents a nonspecific binding in cell culture experiments. This conclusion is in accordance with the histologic finding that IgA deposits are rarely detected in the tubulointerstitium in IgAN [16]. Aberrant glycosylation of O-glycans and the electrostatic interaction due to the anionic nature of the IgA molecule may be operative in the binding of IgA to HMC in IgAN [22, 32]. Increased binding of IgA to various components of ECM had been demonstrated in IgAN [33]. Whether similar mechanisms are operated for the nonspecific binding of IgA to cultured PTEC remain to be explored.

Based on our findings, we propose that tubular activation and interstitial injury in IgAN are not due to the direct exposure of PTEC to IgA within the tubular lumen. Other than the tubulotoxic role of proteinuria, we hypothesize that mediators or soluble factors released from HMC following IgA deposition lead to PTEC activation. The concept that a glomerulotubular cross-talk in IgAN is novel, but communication between compartments of the nephron has been reported by others and us. A tubuloglomerular cross-talk exists during heavy proteinuria, leading to glomerulosclerosis [14]. Recently, mesangial cells have been found to communicate with podocytes through interferon-inducible protein-10 [34]. To confirm our hypothesis, we conducted PTEC culture experiments using spent medium from HMC preincubated with different IgA preparations. This medium transfer setting allowed no direct cell-cell communication but simulated the in vivo glomerulotubular communication via humoral factors. Spent medium from HMC incubated with IgA from patients with IgAN, but not with IgA from MCNS, HSP, or healthy subjects, increased PTEC proliferation. Simultaneously, there was up-regulation in the gene expression and protein synthesis of TNF-α, MIF, and ICAM-1 in PTEC cultured with IgA-HMC spent medium from patients with IgAN. The increased synthesis of TNF-α after culturing with IgA-HMC medium was not due to the 'left over' from the HMC spent medium because there was enhanced gene expression of TNF-α in the PTEC. Moreover, the concentration of TNF-α in the supernatant of cultured PTEC was at least 4-fold higher than that in the IgA-HMC medium. These observations are in line with recent studies reporting an up-regulation of MIF and ICAM-1 expression in the tubular cells from patients with IgAN [35, 36]. Urinary excretion of MIF is increased in patients with IgAN, and the level correlates with the disease activity [37]. Mesangial cells can be activated to produce cytokines and chemokines, including IL-1, IL-6, TNF-α, MCP-1, TGF-β, and PDGF [38, 39]. We speculate that these humoral factors/mediators from mesangial cells may reach the tubulointerstitium either by glomerular filtration or by transportation via the postglomerular capillaries. Upon reaching the tubular compartment, these mediators could stimulate PTEC to produce other proinflammatory cytokines and chemokines that eventually lead to tubular damage, interstitial mononuclear cell infiltration, and fibrosis. One of the possible mediators is TNF-α produced by HMC following stimulation by pIgA from IgAN patients [25]. Our finding of increased synthesis of TNF-α by PTEC exposed to IgA-HMC spent medium suggests an autocrine effect of the cytokine. Moreover, the result from culture experiments using neutralizing anti-TNF-α antibody suggests that TNF-α is a potent mediator, at least for MIF synthesis, in PTEC exposed to IgA-HMC spent medium. The continuous and local synthesis of

these inflammatory cytokines and chemokines plays an important role in the pathogenesis of chronic tubulointerstitial injury in IgAN [40]. Since sICAM-1 synthesis by PTEC exposed to IgA-HMC spent medium was only partially blocked by neutralizing anti-TNF-α antibody, we further examined the synthesis MIF and sICAM-1 by PTEC activated with IgA-HMC spent medium in the presence of other neutralizing antibodies to potential mediators, including IL-1β, TGF-β, or PDGF. As predicted, neutralizing antibodies to IL-1β or PDGF did not reduce the synthesis of MIF or sICAM-1 by PTEC cultured with IgA-HMC spent medium because we had detected no induction of PDGF or IL-1β synthesis in HMC incubated with IgA from patients with IgAN. Anti-TGF-β did not reduce the synthesis of MIF or sICAM-1 by PTEC cultured with IgA-HMC spent medium. This could be because the concentration of TGF-β in the IgA-HMC medium (∼40 pg/mL) is not high enough to elicit these inflammatory responses as observed by other investigators [41]. Taken together, our finding suggests that other mediators released from HMC activated by pIgA may also take part in inducing the tubulointerstitial injury, and further investigation is warranted.

Our experiments showed that spent medium from HMC incubated with IgA from patients with IgAN, but not with IgA from control subjects, increased proliferation and synthesis of TNF-α, MIF, or ICAM-1 in PTEC. Of note is the different finding between IgAN and HSP, despite the latter having similar renal histopathology. This could be due to the difference in the size of circulating IgA immune complexes, the subclass of IgA (λ− versus κ−light chain) deposited in the mesangium, and the regulation of B cells by T lymphocytes between the two glomerulonephritides (review in [42]). The difference in the functional effect on mesangial cells (such as synthesis of MIF and angiotensin II) between polymeric IgA isolated from patients with IgAN and patients with HSP has previously been documented [25, 43].

CONCLUSION

The absence of IgA binding through known IgA receptors, and the lack of PTEC activation, supports the notion that IgA is rarely deposited in the tubulointerstitium in IgAN. However, we have shown that mediators released from HMC after mesangial IgA deposition can activate PTEC and lead to subsequent inflammatory changes in PTEC. Our finding implicates that humoral factors released from glomerular mesangium maintain a glomerulotubular cross-talk in the development of tubulointerstitial damage in IgAN.

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