Polymeric IgA1 from Patients with IgA Nephropathy Upregulates Transforming Growth Factor-β Synthesis and Signal Transduction in Human Mesangial Cells via the Renin-Angiotensin System

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Abstract. The effects of polymeric IgA1 (pIgA1) and monomeric IgA1 (mIgA1) from patients with IgA nephropathy (IgAN) on the renin-angiotensin system (RAS) and TGF-β synthesis were examined in cultured human mesangial cells (HMC). Both pIgA1 and mIgA1 induced renin gene expression in HMC, in a dose-dependent manner. Similar findings were observed for TGF-β gene and protein expression. The values measured in HMC incubated with pIgA1 were significantly higher than those in HMC incubated with equivalent amounts of mIgA1. When similar experiments were performed with the addition of either captopril or losartan, there was a significant increase in the renin gene expression by HMC, whereas the synthesis of TGF-β was markedly reduced. The TGF-β signal transduction pathways in HMC were studied by measuring the receptor-regulated Smad proteins (Smad 2 and 3) and common-partner Smad proteins (Smad 4). pIgA1 from patients with IgAN upregulated Smad activity in HMC, and the activity observed in HMC that had been preincubated with pIgA1 was readily suppressed with optimal concentrations of captopril or losartan. The effects of pIgA1 on the RAS were further examined in HMC incubated with IgA isolated from 30 patients with IgAN, 30 healthy subjects, and disease control subjects with other diseases. pIgA1 induction of angiotensin II or TGF-β synthesis in HMC was significantly greater with preparations from patients with IgAN, compared with healthy or disease control subjects. The findings support a pathogenetic role of pIgA1 in IgAN through upregulation of the RAS and TGF-β, leading to chronic renal failure with renal fibrosis.

IgA nephropathy (IgAN), the most common glomerulonephritis throughout the world, exhibits an indolent but slowly progressive course, leading to end-stage renal failure in 30 to 40% of patients more than 30 yr of age (1). The disease is characterized by mesangial deposition of polymeric IgA1 (pIgA1), proliferation of mesangial cells, increased synthesis of extracellular matrix, and infiltration by macrophages, monocytes, and T cells (2). Immunoregulatory abnormalities involving IgA1 synthesis in IgAN include overproduction of IgA1 by B lymphocytes in vitro (3,4), increased serum levels of IgA, IgA-containing immune complexes (5,6), and anionic IgA (7), and abnormal glycosylation of the carbohydrate moieties in IgA1 from these patients (8). Five different IgA receptors have been identified in human subjects, including FcαR1 or CD89, the polymeric Ig receptor, the asialoglycoprotein receptor, FcαR, and the transferrin receptor or CD71. The expression of these receptors in mesangial cells and their binding to IgA remain uncertain. In addition, a genome-wide analysis indicated the linkage of IgAN with chromosome 6q22–23 (9). The pathogenesis of IgAN is not completely resolved, however, because none of these abnormalities can adequately explain how IgA deposits in the glomerular mesangium lead to progressive renal injury. Recent data suggested that serum IgA from patients with IgAN is different from that of healthy subjects and can exert pathophysiologic effects on target cells (10,11). However, there is little information regarding the stimulatory effects of IgA on the release of “sclerogenic” growth factors from mesangial cells, which lead to excess matrix formation and glomerulosclerosis.

The renin-angiotensin system (RAS) has been recognized as a key factor in the progression of chronic renal failure. Angiotensin II (AngII) plays a central role as a mediator of glomerular hemodynamic adaptation and injury. It has been suggested that AngII-induced mesangial cell contraction with efferent arteriolar vasoconstriction initiates intraglomerular hypertension and may eventually lead to enhanced matrix formation and renal fibrosis after increased synthesis of TGF-β (12). The beneficial effects of either angiotensin-converting enzyme (ACE) inhibitors (ACEI) or AngII subtype 1 (AT1) receptor

Received May 6, 2003. Accepted August 18, 2003.
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Journal of the American Society of Nephrology
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DOI: 10.1097/01.ASN.0000095639.56212.BF
antagonists on proteinuria and creatinine clearance (independent of BP reduction) highlight the importance of this system in IgAN (13,14). In this study, we explore whether plgA1 isolated from patients with IgAN exerts any stimulatory effect on the RAS of mesangial cells, resulting in increased synthesis of TGF-β.

Materials and Methods

Patient Consent and Materials

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee for studies with human subjects. All subjects (patients and healthy control subjects) gave their written informed consent for serum and tissue collections.

RPMI 1640 medium and FCSs were obtained from Life Technologies (Rockville, MD). Jacalin-agarose was obtained from Pierce (Rockford, IL). The standard and antibodies for the TGF-β ELISA were obtained from R & D Systems (Minneapolis, MN). The Superose fast protein liquid chromatography (FPLC) column was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Consumables for electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Human Mesangial Cell Cultures

Isolation and characterization of human mesangial cells (HMC) were performed as previously described (15). Glomeruli were prepared from the cortex of a human cadaveric kidney judged to be unsuitable for transplantation or from the intact pole of a kidney removed for treatment of a circumscribed tumor. Histologic examinations of the kidney samples revealed no renal pathologic conditions. Glomerular cells were grown in RPMI 1640 medium supplemented with glucose (2 mM), HEPES (10 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and 12% FCS, in an atmosphere of 5% CO₂/95% air. Mesangial cells have a stellate appearance and grow in clumps. They exhibit a network of intracellular myosin fibrils, and they contract in the presence of 1 nM AngII. Mesangial cells from a single nephrectomy sample at passage 4 to 7 were used in our experiments.

Purification and Characterization of plgA1 and Monomeric IgA1 with Jacalin-Agarose Affinity Chromatography and FPLC

IgA1 was purified, with a jacalin-agarose affinity column, from five randomly selected patients with mild IgAN (grade 1) in clinical quiescence. The severity of the renal pathologic condition was classified as grade 1, 2, or 3, as described previously (16); grade 1 indicated mesangial proliferation with no crescents, sclerosis, or tubulointerstitial changes. These five patients exhibited normal creatinine clearance values (>80 ml/min per 1.73 m²), with proteinuria of <0.5 g/dl. IgA1 was fractionated at room temperature with an FPLC system (Pharmacia, Uppsala, Sweden), as previously described (15). Briefly, monomeric IgA1 (mlgA1) and plgA1 were separated with FPLC after jacalin-agarose affinity chromatography. The identity of IgA1 after FPLC was confirmed with immunoblotting and SDS-polyacrylamide gel electrophoresis. Every IgA preparation from each patient was separated into mlgA1 and plgA1 according to molecular mass. Chromatography fractions with molecular masses of 300 to 1000 kD were considered low-molecular mass IgA or mlgA1. Distinct mlgA1 and plgA1 fractions were separately prepared from each patient for subsequent incubation studies. The IgG contents in the fractions were measured with an anti-IgG ELISA. The fractions were dialyzed, concentrated to 2 ml with Centriprep cartridges (Amicon, Beverly, MA), and stored at -70°C until use. The purity of the IgA1 fractions was confirmed with Coomassie blue and silver staining after SDS-polyacrylamide gel electrophoresis and ELISA (15). The endotoxin levels in the IgA preparations were determined with the QCL-1000 Limulus amebocyte lysate kit (BioWhittaker, Walkersville, MD). The levels in all preparations were <10 EU/ml.

Treatment of HMC with Different IgA Preparations or RAS Blockers

HMC were grown to logarithmic phase and were harvested with 0.05% trypsin. The trypsin-recovered cells were subcultured onto six-well culture plates (1 × 10⁶ cells/well). The cells were washed with 24 h of growth in RPMI 1640 medium with 0.5% FCS, and that medium (with 0.5% FCS) was used for all subsequent experiments. The cells were exposed to IgA preparations or RAS blockers at increasing concentrations (0, 0.125, 0.25, 0.5, 1, or 2 mg/ml for IgA and 0, 1, 10, 100, 1000, and 10000 nM for captopril or losartan) for 6 or 24 h at 37°C, in replicate. Preliminary experiments demonstrated that these incubation periods were convenient and were associated with optimal synthesis of mRNA for renin or TGF-β (6 h) and protein for TGF-β (24 h). Cells were harvested for RNA extraction and nuclear extract preparation. Culture supernatants were collected for determination of TGF-β and/or AngII levels.

RNA Extraction, cDNA Synthesis, and Renin/TGF-β Gene Expression in Cultured HMC

Specific primers for renin, TGF-β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed on the basis of known GenBank sequences (renin, accession number XM_010626; TGF-β1, accession number X02812; GAPDH, accession number X01677). The sequences of the primer pairs were as follows: renin, sense primer, 5'-TCAATGACTGGTGTGGC-3'; antisense primer, 5'-GGGC-CAGGCCGAAGGCCA-3'; TGF-β, sense primer, 5'-GCGCTGGA-CAACCAATTTGCT-3'; antisense primer, 5'-AGGCCCTCAATG-TAGGGGAGG-3'; GAPDH, sense primer, 5'-ACGACGCTCAT-GGCCATCAC-3'; antisense primer, 5'-TCCAACACCCTGGTCTGTA-3'. We performed reverse transcription-PCR as previously described (17), using the following profile: first cycle, 94°C for 3 min, 55°C for 1 min, and 72°C for 1 min; cycles 2 to 30, 95°C for 45 s, 55°C for 40 s, and 72°C for 45 s; final cycle, 94°C for 1 min and 72°C for 10 min. The PCR products from the renin or TGF-β and control (GAPDH) amplicons were mixed, separated on 1.5% (wt/vol) agarose gels, and stained with ethidium bromide, and the gel images were captured and analyzed with the Gel Doc 1000 gel documentation system and Quantity One software (Bio-Rad Laboratories, Hercules, CA). We semiquantitatively assessed the renin and TGF-β mRNA yields as renin/GAPDH and TGF-β/GAPDH ratios. We took all
necessary steps to ensure the validity of the results, as described previously (18).

**Determination of TGF-β and AngII Levels in Culture Supernatants**

A sandwich ELISA (R & D Systems) was used to measure TGF-β levels in the culture supernatants. The ELISA for TGF-β measured both the active and latent forms. The sensitivity limit was 5 pg/ml. All samples were assayed at the same time, to avoid interbatch variations. The intra-assay coefficient of variation was <7.3% (19). AngII levels were measured with an enzyme immunoassay using an anti-AngII Fab’ mAb labeled with acetylcholinesterase (20) (SPI Bio, Massy Cedex, France). The minimal detectable concentration was 1 pg/ml, and the intra-assay coefficient of variation was 7%.

**Determination of Effects of IgA Treatment or RAS Blockade on Smad Activation by TGF-β in Cultured HMC**

Smad activation in HMC with IgA stimulation, with or without RAS blockade, was examined with an electrophoretic mobility shift assay, performed as described previously (21). Briefly, 32P-labeling of Smad oligonucleotides (5'-TTTCTCAGACAGACTGTCTGAGAAA-3' and 5'-GGCAGACAGACTGTCTGAGAAA-3') (22) was performed with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (300 Ci/mmol). The single-stranded oligonucleotides were annealed for 2 h while the temperature was decreased from 95°C to 25°C. Labeled DNA was separated from unincorporated radioactivity. Nuclear extracts were prepared with a kit for the isolation of nuclei, according to the instructions provided by the manufacturer (Sigma). Binding reactions were performed by adding 2 µg of nuclear protein to 20 µl of binding buffer containing [γ-32P]ATP-labeled Smad oligonucleotide probes. Samples were incubated at room temperature for 25 min, fractionated by electrophoresis, transferred to 3 MM paper, dried, and exposed to Hyperfilm-MP x-ray film at −70°C, with an intensifying screen. The results were quantified with a scanning densitometer. To assess the specific binding to nuclear extracts of the Smad probes, competitive assays were performed with a 100-fold molar excess of unlabeled oligonucleotides. Where indicated, supershift assays were performed with antibodies against Smad 2, 3, and 4 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Confirmation that plgA1 from Patients with IgAN Exerts Upregulatory Effects on the RAS in HMC**

The stimulatory effects of plgA1 from patients with IgAN on the RAS in HMC were further studied with 30 Chinese patients (17 male and 13 female patients; age range, 24 and 61 yr; mean age, 39 yr) with clinical and renal immunopathologic diagnoses of primary IgAN. The histologic diagnoses had been made at least 18 mo before the study, and the serum creatinine levels of the patients had remained stable with respect to age and race, with no microscopic hematuria or proteinuria, were used as healthy control subjects. Patients with Henoch-Schönlein purpura, lupus nephritis, minimal-change nephropathy, or membranous nephropathy were recruited as disease control subjects. Serum samples were similarly collected from those individuals for IgA purification.

**Statistical Analyses**

All data (for patients or cell culture experiments) were expressed as means ± SD. Intergroup differences for continuous variables were assessed with the unpaired t test, except for concentrations exceeding the in vivo relevance (for plgA1) or the therapeutic level (for captopril or losartan). The renin/TGF-β mRNA expression and TGF-β protein synthesis in cultured cells after exposure to different concentrations of IgA preparations or AngII blockers were analyzed with multivariate ANOVA for repeated measures. All P values presented are two-tailed, and significance was defined as P < 0.05.

**Results**

**Expression of Renin mRNA in Cultured HMC**

Inhibition of ACE after incubation of the HMC with captopril led to increased renin gene expression in a dose-dependent manner, peaking at a concentration of 1000 nM (Figure 1). Blockade of AT1 receptors also resulted in a stepwise increase in renin mRNA expression.

![Figure 1. Upregulation of renin mRNA in cultured human mesangial cells (HMC) incubated with captopril, losartan, or IgA1 preparations from patients with IgA nephropathy (IgAN) (P < 0.0001, multivariate ANOVA). Measurements of renin mRNA levels in HMC incubated with captopril at different concentrations differed significantly (P < 0.01). Measurements of renin mRNA levels in HMC incubated with losartan at different concentrations differed significantly (P < 0.05). Measurements of renin mRNA levels in HMC incubated with plgA1 at different concentrations differed significantly (P < 0.01). Measurements of renin mRNA levels in HMC incubated with plgA1 differed at different concentrations differed significantly (P < 0.01). Measurements of renin mRNA levels in HMC incubated with plgA1 differed at different concentrations differed significantly (P < 0.01).](image-url)

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in renin gene expression in HMC, but the effect peaked at a lower concentration of 100 nM. At comparable molar concentrations, the stimulatory effect of the ACEI (captopril) was stronger than that of the AT₁ receptor antagonist (losartan) \( (P < 0.001) \). More interestingly, plgA1 from patients with IgAN also significantly upregulated renin gene expression in a dose-dependent manner, reaching 83 and 151\% increases at concentrations of 0.5 and 2 mg/ml, respectively (Figure 1). Although mlgA1 from patients with IgAN upregulated renin gene expression in a similar, dose-dependent manner, the magnitude of the effect was smaller; renin gene expression in HMC increased by only 22 and 54\% with mlgA1 at concentrations of 0.5 and 2 mg/ml, respectively.

Expression of TGF-β mRNA and Protein in Cultured HMC

With decreased binding of AngII to its receptors on HMC, produced with either reduction of AngII synthesis with captopril or blockade of AT₁ receptors with losartan, TGF-β gene expression was decreased in a dose-dependent manner, rapidly reaching a trough at concentrations between 1 and 10 nM (Figure 2a). In contrast, plgA1 from patients with IgAN significantly upregulated TGF-β gene expression in a dose-dependent manner, with 138 and 213\% increases at concentrations of 0.5 and 2 mg/ml, respectively (Figure 2a). Again, mlgA1 from patients with IgAN upregulated TGF-β gene expression in a similar, dose-dependent manner, but to a lesser extent. TGF-β gene expression in HMC was increased by only 43 and 73\% with mlgA1 at concentrations of 0.5 and 2 mg/ml, respectively.

The findings regarding TGF-β protein levels in supernatants from cultured HMC paralleled those regarding TGF-β mRNA levels. Incubation with captopril or losartan decreased TGF-β protein synthesis by HMC in a dose-dependent manner, rapidly reaching a trough at concentrations between 1 and 10 nM (Figure 2b). Again, plgA1 from patients with IgAN significantly upregulated TGF-β protein synthesis in a dose-dependent manner, reaching 51 and 122\% increases at concentrations of 0.5 and 2 mg/ml, respectively. mlgA1 from patients with IgAN upregulated TGF-β protein synthesis to a lesser extent; TGF-β gene expression in HMC was increased by only 26 and 44\%, respectively.

Exposure of cultured HMC to AngII increased TGF-β synthesis in a dose-dependent manner (Figure 3). The TGF-β synthesis induced by preincubation with AngII was significantly decreased in the presence of losartan (100 nM), reaching 63 and 75\% reductions at concentrations of 0.1 and 0.01 nM, respectively. A similar reduction of TGF-β synthesis induced by AngII was not observed in HMC exposed to captopril (data not shown).

Stimulatory Effects of IgA on TGF-β Synthesis in Cultured HMC Preincubated with Optimal Doses of an ACEI or a AT₁ Receptor Antagonist

We then investigated the stimulatory effects of IgA (from patients with IgAN) on TGF-β synthesis in cultured HMC that had been preincubated with an optimal dose of captopril or losartan or IgA1 preparations from patients with IgAN (\( P < 0.0001 \), multivariate ANOVA). Measurements of TGF-β mRNA levels in HMC incubated with captopril at 0 and 1 nM differed significantly from measurements at higher concentrations \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with losartan at different concentrations were significantly lower than the baseline measurements without losartan \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with plgA1 at different concentrations differed significantly \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with mlgA1 at different concentrations differed significantly, except for measurements at 0 and 0.125 mg/ml and those at 0.25 and 0.5 mg/ml \( (P < 0.05) \). The results represent the mean ± SD of five separate experiments.

Figure 2. (a) Effects on TGF-β mRNA expression in cultured HMC incubated with captopril, losartan, or IgA1 preparations from patients with IgAN \( (P < 0.0001 \), multivariate ANOVA). Measurements of TGF-β mRNA levels in HMC incubated with captopril at 0 and 1 nM differed significantly from measurements at higher concentrations \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with losartan at different concentrations were significantly lower than the baseline measurements without losartan \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with plgA1 at different concentrations differed significantly \( (P < 0.01) \). Measurements of TGF-β mRNA levels in HMC incubated with mlgA1 at different concentrations differed significantly, except for measurements at 0 and 0.125 mg/ml and those at 0.25 and 0.5 mg/ml \( (P < 0.05) \). The results represent the mean ± SD of five separate experiments. (b) Effects on TGF-β protein synthesis in cultured HMC incubated with captopril, losartan, or IgA1 preparations from patients with IgAN \( (P < 0.0001 \), multivariate ANOVA). Measurements of TGF-β levels in supernatants from HMC incubated with captopril at 0 and 1 nM differed significantly from measurements at higher concentrations \( (P < 0.01) \). Measurements of TGF-β levels in supernatants from HMC incubated with losartan at different concentrations were significantly lower than the baseline measurements without losartan \( (P < 0.01) \). Measurements of TGF-β levels in supernatants from HMC incubated with plgA1 at different concentrations differed significantly, except for measurements at 0 and 0.125 mg/ml and those at 0.25 and 0.5 mg/ml \( (P < 0.05) \). The results represent the mean ± SD of five separate experiments.
losartan for 4 h. Under baseline conditions, captopril at a concentration of 100 nM significantly reduced TGF-β gene expression (0.243 ± 0.015 versus 0.266 ± 0.019, \( P < 0.005 \)) and TGF-β synthesis (250.6 ± 10.0 versus 336.1 ± 36.8 pg/ml, \( P < 0.0001 \)), compared with growth-arrested HMC incubated with culture medium alone (Figure 4). Similarly, losartan at a concentration of 100 nM significantly reduced TGF-β gene expression (0.226 ± 0.013 versus 0.266 ± 0.019, \( P < 0.0001 \)) and TGF-β synthesis (226.3 ± 13.1 versus 336.1 ± 36.8 pg/ml, \( P < 0.0001 \)), compared with growth-arrested HMC. However, plgA isolated from patients with IgAN was able to reverse part of the suppressive effect of RAS blockade on TGF-β synthesis, in a dose-dependent manner (Figure 4). The antagonistic effect of plgA on the suppression of TGF-β synthesis with RAS blockade was significantly smaller in HMC that had been pretreated with losartan than in HMC that had been pretreated with captopril. These findings support a better suppressive effect on TGF-β synthesis of losartan, compared with captopril, at comparable molar concentrations.

Effects of RAS Blockade in Cultured HMC Preincubated with IgA

Next we preincubated HMC with an optimal dose of IgA for 4 h, to stimulate the RAS. plgA (0.5 mg/ml) produced greater renin gene expression than did mlgA, at a equivalent concentration, or control medium (\( P < 0.0001 \)) (Figure 5a). The renin mRNA levels were further increased when HMC that had been preincubated with plgA were treated with captopril (100 nM), reaching levels similar to those in HMC treated with captopril alone (100 nM). However, a further increase in renin mRNA levels was not observed when HMC that had been preincubated with plgA were treated with losartan (100 nM). Parallel to the findings regarding renin gene expression, plgA induced greater AngII synthesis than did mlgA, at an equivalent concentration, or control medium (\( P < 0.0001 \)) (Figure 5b). However, AngII synthesis was significantly decreased when HMC that had been preincubated with plgA were treated with captopril or losartan (100 nM). Similarly, the TGF-β synthesis induced by plgA was significantly greater than that induced by mlgA or...
bation with mIgA1 (0.5 mg/ml).

Incubation with losartan (100 nM) downregulated the synthesis of AngII in HMC induced by preincubation with pIgA1. Incubation with losartan (100 nM) decreased the synthesis of TGF-β in HMC incubated with mIgA1 alone. Incubation with captopril (100 nM) or losartan (100 nM) upregulated the renin gene expression in cultured HMC that had been preincubated with pIgA1 (0.5 mg/ml). Similarly, TGF-β gene expression induced by pIgA1 was significantly greater than that induced by mIgA1 or control medium (P < 0.0001) (Figure 5c). The TGF-β levels decreased significantly when HMC that had been preincubated with pIgA1 were treated with captopril (100 nM). However, an additional decrease in TGF-β levels in the supernatant was observed when HMC that had been preincubated with pIgA1 were treated with a comparable molar concentration of losartan (100 nM). Similarly, TGF-β gene expression induced by pIgA1 was significantly greater than that induced by mIgA1 or control medium (P < 0.0001) (data not shown). Again, the TGF-β mRNA levels were significantly decreased when HMC that had been preincubated with pIgA1 were treated with captopril or losartan (data not shown).

Identical experiments performed with mlgA1 demonstrated similar patterns for renin gene expression and AngII and TGF-β protein synthesis, but the magnitude of the stimulatory effect was smaller than with pIgA1. For HMC cultured with control medium, the addition of captopril or losartan enhanced renin gene expression (Figure 5a). This was associated with a mild reduction in AngII synthesis (for losartan) and an insignificant decrease in TGF-β expression (Figure 5, b and c).

Confirmation that pIgA1 from Patients with IgAN Exerts Upregulatory Effects on the RAS in HMC

The stimulatory effects of pIgA1 from patients with IgAN on the RAS in HMC were investigated for 30 patients, compared with 30 healthy control subjects comparable with respect to age and gender. The serum IgA levels among patients with IgAN (2.94 ± 1.44 g/L) were significantly higher than those among healthy control subjects (1.88 ± 0.89 g/L, P < 0.001). No IgG or IgM was detected in the mlgA1 fractions and IgG represented 0.1% of protein in total IgA1 fractions, as measured by ELISA. Studies of IgA in the FPLC fractions with IgA ELISA demonstrated that mlgA1 amounted to 90% of total IgA1 from either control subjects or patients (data not shown).

HMC incubated with equivalent concentrations of pIgA1 from patients or control subjects exhibited significantly greater renin gene expression, compared with cells incubated with culture medium alone (Figure 6a). A smaller but significant stimulatory effect was observed with mlgA1 isolated from patients but not healthy control subjects. Although the IgA was isolated during clinical quiescence, pIgA1 from patients induced significantly greater renin gene expression (23%) than did pIgA1 from healthy control subjects (P < 0.0001). Similarly, pIgA1 from patients induced greater protein synthesis of AngII (56%) and TGF-β (37%) than did pIgA1 from healthy preparations. Incubation with captopril (100 nM) or losartan (100 nM) downregulated the synthesis of TGF-β in HMC induced by preincubation with pIgA1. *P < 0.01, compared with HMC incubated with pIgA1 alone. *P < 0.05, compared with pIgA1-treated HMC that were subsequently incubated with captopril. Incubation with captopril (100 nM) or losartan (100 nM) upregulated the renin gene expression in HMC that had been preincubated with mlgA1 (0.5 mg/ml). A similar effect was not observed with losartan (100 nM). *P < 0.05, compared with mlgA1-treated HMC that were subsequently incubated with captopril. Incubation with captopril (100 nM) or losartan (100 nM) upregulated the renin gene expression in HMC that had been preincubated with pIgA1 (0.5 mg/ml). †P < 0.05, compared with HMC incubated with mlgA1 alone. ‡P < 0.001, compared with mlgA1-treated HMC that were subsequently incubated with captopril. The results represent the mean ± SD of five separate experiments.

**Figure 5.** (a) Renin gene expression after renin-angiotensin system (RAS) blockade in cultured HMC that had been preincubated with IgA1 preparations. Incubation with captopril (100 nM) or losartan (100 nM) upregulated the renin gene expression in resting HMC. *P < 0.05, compared with growth-arrested HMC. **P < 0.001, compared with HMC incubated with losartan. Incubation with captopril (100 nM) further upregulated the renin gene expression in HMC that had been preincubated with pIgA1 (0.5 mg/ml). A similar effect was not observed with losartan (100 nM). *P < 0.05, compared with pIgA1-treated HMC that were subsequently incubated with captopril. Incubation with captopril (100 nM) or losartan (100 nM) upregulated the renin gene expression in HMC that had been preincubated with mlgA1 (0.5 mg/ml). †P < 0.05, compared with HMC incubated with mlgA1 alone. ‡P < 0.001, compared with mlgA1-treated HMC that were subsequently incubated with captopril. The results represent the mean ± SD of five separate experiments. (b) Effects of RAS blockade on AngII synthesis in cultured HMC that had been preincubated with IgA1 preparations. Incubation with losartan (100 nM) decreased the synthesis of AngII by resting HMC. *P < 0.05, compared with growth-arrested HMC. Incubation with captopril (100 nM) or losartan (100 nM) downregulated the synthesis of AngII in HMC induced by preincubation with pIgA1 (0.5 mg/ml). †P < 0.05, compared with HMC incubated with pIgA1 alone. Incubation with losartan (100 nM) downregulated the synthesis of AngII in HMC induced by preincubation with mlgA1 (0.5 mg/ml). †‡P < 0.05, compared with HMC incubated with mlgA1 alone. The results represent the mean ± SD of five separate experiments. (c) Effects of RAS blockade on TGF-β synthesis in cultured HMC that had been preincubated with IgA1 preparations. Incubation with captopril (100 nM) or losartan (100 nM) downregulated the synthesis of TGF-β in HMC induced by preincubation with pIgA1. *P < 0.01, compared with HMC incubated with pIgA1 alone. *P < 0.05, compared with pIgA1-treated HMC that were subsequently incubated with captopril. Incubation with captopril (100 nM) or losartan (100 nM) downregulated the synthesis of TGF-β in HMC induced by preincubation with mlgA1 (0.5 mg/ml). †P < 0.05, compared with HMC incubated with mlgA1 alone. The results represent the mean ± SD of five separate experiments.
control subjects ($P < 0.0001$) (Figure 6, b and c). mIgA1 from patients also induced greater AngII and TGF-β protein synthesis than did mIgA1 from healthy control subjects ($P < 0.0001$).

HMC were incubated with pIgA1 from patients in various disease groups, to explore whether IgA preparations from patients with other glomerulonephritides would also upregulate the RAS in HMC. As indicated in Figure 7, there were significant increases in AngII and TGF-β production after culturing of HMC with pIgA1 from patients with quiescent IgAN, compared with healthy control subjects or disease control subjects, including subjects with Henoch-Schönlein purpura, lupus nephritis, minimal-change nephropathy, or membranous nephropathy. In contrast, AngII and TGF-β production after culturing of HMC with pIgA1 from patients with IgAN and disease control subjects were in periods of clinical quiescence. pIgA1 from patients with IgAN induced greater production of AngII or TGF-β by HMC than did pIgA1 from healthy or disease control subjects. *$P < 0.01$, **$P < 0.02$, compared with HMC incubated with pIgA1 from patients with IgAN. HSP, Henoch-Schönlein purpura; MCN, minimal-change nephropathy; MGN, membranous nephropathy. The results represent the mean ± SD.

**Effects of IgA Treatment or RAS Blockade on TGF-β Signal Transduction in Cultured HMC**

Lastly, we determined whether IgA treatment or RAS blockade affected transcripational control by the TGF-β/Smad signaling system in HMC. Treatment with either captopril or losartan led to a significant decrease in Smad activity in HMC ($P < 0.0001$) (Figure 8). Compared with resting HMC, incubation with pIgA1 from patients with IgAN significantly induced the Smad activity by 110% (2127.2 ± 329.2 versus 1014.0 ± 85.9 units, $P < 0.0001$). In contrast, incubation with mIgA1 from patients with IgAN induced an insignificant increase in the Smad activity, compared with resting HMC (1426.2 ± 342.3 versus 1014.0 ± 85.9 units, $P = 0.058$). Blockade of the RAS with either captopril or losartan at 100 nM effectively reduced the Smad activity in HMC induced by pretreatment with pIgA1 from patients with IgAN ($P < 0.0001$). Supershift
expression of the nuclear transcription factor NFkB revealed that binding of IgA to mesangial cells led to increased dose-dependent increase in calcium flux. Other mesangial cells incubated with aggregated IgA exhibited a rosis through induction of TGF-β. AngII plays a pivotal role in glomerulosclerosis in diabetic and nondiabetic nephropathy (30,31). AngII stimulates extracellular matrix protein synthesis, increases matrix protein receptor levels, and alters protease/protease inhibitor balances, thus inhibiting matrix degradation. TGF-β stimulates extracellular matrix protein synthesis, increases matrix protein receptor levels, and alters protease/protease inhibitor balances, thus inhibiting matrix degradation.

In addition to the local synthesis of TGF-β by mesangial cells, there is increased TGF-β gene expression in circulating CD4+ T cells from patients with IgAN (2). Despite the presence of genes coding for renin, AngII, and ACE in cultured mesangial cells and in mesangial cells in kidney tissues from patients with IgAN (35), information regarding the RAS in IgAN remains scarce and is mainly limited to ACE-related polymorphism. To date, data on the pathogenetic role of different genotypes in IgAN remain divergent and inconclusive (36).

In this study, we set out to explore the effects of IgA isolated from patients with IgAN on the local RAS in HMC. Mesangial cells from a single nephrectomy sample without IgAN were used, because the phenotype of mesangial cells is unlikely to have an effect on the pathogenesis of the nephropathy. This is supported by the observation of early IgA deposition and frequent recurrence of IgAN in renal allografts. Despite lower concentrations of IgA in serum (10 to 13% of total IgA), mesangial IgA deposits in IgAN are predominantly of the A1 subclass and polymeric in nature. IgA represents 64% of the total eluted IgA in glomeruli from patients with IgAN (37). In vitro studies revealed that IgA1 has at least 10-fold higher affinity for HMC than does IgM (26,38). Therefore, we separated isolated IgA1 and IgM from the serum of our patient, to study the time- and dose-related effects of different IgA fractions on the RAS in HMC. For subsequent inhibitory studies and comparisons between patients and control subjects, a concentration of 0.5 mg/ml was used for all IgA preparations in cell cultures. This is comparable to the IgA concentration (i.e., 0.35 mg/ml) in the serum of patients with IgAN, who exhibit an average serum total IgA concentration of 3 g/L, of which 10 to 13% is IgA1.

In resting HMC, incubation with either an ACEI (captopril) or an AT1 receptor antagonist (losartan) resulted in increased renin gene expression, in a dose-dependent manner. Renin mRNA levels are elevated via a negative feedback loop attributable to reduced AngII availability after ACE inhibition or AT1 receptor blockade. The renin gene expression in HMC peaked at different concentrations of captopril and losartan, reflecting the difference in the effectiveness of reducing AngII synthesis with inhibition of the rate-determining enzyme, ACE, or with AT1 receptor blockade. With decreased binding of AngII to its receptors on HMC, produced with either reduction of AngII synthesis with an ACEI or blockade of AT1 receptors with an AT1 receptor antagonist, both TGF-β gene expression and TGF-β protein synthesis were decreased in a dose-dependent manner, rapidly reaching a trough at concentrations between 1 and 10 nM. More interestingly, IgA from patients with IgAN also significantly upregulated renin gene expression in a dose-dependent manner, producing 83 and 151% increases at concentrations of 0.5 and 2 mg/ml, respectively. IgA from patients with IgAN also upregulated renin gene expres-

The RAS has been implicated in the development of progressive glomerulosclerosis in diabetic and nondiabetic nephropathy (30,31). AngII plays a pivotal role in glomerulosclerosis through induction of TGF-β expression in mesangial cells (32). TGF-β stimulates extracellular matrix protein synthesis, increases matrix protein receptor levels, and alters protease/protease inhibitor balances, thus inhibiting matrix degradation. TGF-β induces tubular epithelial cell-myofibroblast trans-differentiation in vitro, which supports its involvement in the development of glomerular and tubular fibrosis (33,34). In addition to the local synthesis of TGF-β by mesangial cells, there is increased TGF-β gene expression in circulating CD4+ T cells from patients with IgAN (2). Despite the presence of genes coding for renin, AngII, and ACE in cultured mesangial cells and in mesangial cells in kidney tissues from patients with IgAN (35), information regarding the RAS in IgAN remains scarce and is mainly limited to ACE-related polymorphism. To date, data on the pathogenetic role of different genotypes in IgAN remain divergent and inconclusive (36).

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sion in HMC, but to a lesser extent. The increased renin mRNA levels could be either secondary to reduced AngII levels (negative feedback control) or attributable to a direct stimulatory effect of pIgA1 on renin synthesis. Our findings on AngII levels in the supernatants clearly indicated that pIgA1 from patients with IgAN upregulated the RAS in HMC. The increased AngII synthesis further upregulated TGF-β gene expression and TGF-β protein synthesis, in a dose-dependent manner. This concept was supported by the finding that part of the suppressive effect of RAS blockade on TGF-β synthesis was reversed by pIgA1 isolated from patients with IgAN. The effective therapeutic serum concentrations in human subjects for captopril and losartan were 230 nM (50 ng/ml) (39) and 280 nM (120 ng/ml) (40), respectively, after oral administration of captopril (25 mg) or losartan (50 mg), whereas the peak concentrations for captopril and losartan were 1220 nM (41) and 700 nM (42), respectively. Patients with IgAN demonstrated comparable decreases in BP and proteinuria with such therapeutic regimens (captopril, 25 mg, twice daily, or losartan, 50 mg, once daily) (43). Because the tissue concentrations of both compounds may be lower than the serum concentrations, concentrations of 100 nM for captopril and losartan were chosen for our inhibitory studies of pIgA1-induced AngII and TGF-β synthesis in HMC. Our study also suggested that losartan, at a comparable molar concentration, was more potent than captopril in reducing TGF-β synthesis in HMC.

Our initial in vitro studies were performed with IgA isolated from five randomly selected patients with IgAN of mild histologic severity, during clinical quiescence. The stimulatory effects of pIgA1 from patients with IgAN on the RAS in HMC were confirmed with another 30 patients with IgAN of various histologic grades. pIgA1 from patients with IgAN upregulated renin gene expression and enhanced AngII and TGF-β synthesis in HMC, compared with cells incubated with pIgA1 from healthy or disease control subjects with different glomerulonephritides. The upregulatory effects of pIgA1 from patients with IgAN on the RAS in HMC demonstrated in this study and the recent finding of increased nitric oxide generation with RAS blockade among patients with IgAN but not healthy subjects (44) reflect the importance of the RAS in IgAN. We recently demonstrated that pIgA1 from patients with IgAN but not from patients with other nephropathies was capable of inducing macrophage migration inhibitory factor and TNF-α production in HMC (45). Our observation that the upregulatory effects of pIgA1 on the RAS in HMC were not noted with pIgA1 from patients with other glomerulonephritides provides further strong support for the unique pathogenetic role of pIgA1 in IgAN.

TGF-β family members are multifunctional hormones, with the nature of their effects depending on the type and state of the cells. Interestingly, a rather simple system mediates many diverse TGF-β responses. This system involves a family of membrane receptor protein kinases and a family of receptor substrates, the Smad proteins, which move into the nucleus, where they act as transcription factors (46). The Smad proteins are the only TGF-β receptor substrates with a demonstrable ability to propagate signals. Recent studies demonstrated that blockade of TGF-β signaling in T cells prevents the development of experimental glomerulonephritis (47) and blockade of Smad 2 activation inhibits the fibrotic effect of TGF-β on renal tubular epithelial cells (34). This study demonstrated that reduction of AngII binding to AT₁ receptors in HMC with either an ACEI or an AT₁ receptor antagonist led to significant decreases in the expression of receptor-regulated (Smad 2 and 3) or common-partner (Smad 4) Smad forms in HMC. More intriguingly, Smad activity was upregulated in HMC incubated with pIgA1 from patients with IgAN. The Smad activity observed in HMC that had been preincubated with pIgA1 was readily suppressed with optimal concentrations of the ACEI or AT₁, receptor antagonist. These findings support the idea that, in IgAN, pIgA1 upregulates TGF-β synthesis and signal transduction in HMC via the RAS.

Our in vitro findings have interesting relevance to the therapeutic approach to IgAN. pIgA1 directly stimulates the RAS in HMC, which in turn activates the synthesis of TGF-β, leading to extracellular matrix formation and accumulation, tubular epithelial cell-myofibroblast trans-differentiation, and renal fibrosis. The in vitro studies demonstrated that blockade of the RAS, with either reduced AngII synthesis or decreased binding of AngII to AT₁ receptors, suppressed the synthesis of TGF-β, preventing the development of renal fibrosis. The in vitro upregulation of renin and AngII by pIgA1 in HMC suggests that early aggressive blockade of the RAS is essential for preventing the progression of renal deterioration in IgAN. In conclusion, pIgA1 from patients with IgAN plays a significant pathogenetic role in glomerular and tubular sclerosis, by inducing TGF-β synthesis in HMC via direct stimulation of the RAS.

Acknowledgments

This study was supported by a Merck Medical School grant (Grant HK-03-01-LAI) and Research Grant Council (Hong Kong) Grant HKU 7263/01M. Ms. Chan was supported by the Fresnius Medical Service, and Dr. Leung was supported by the Mr. and Mrs. Liu Lit-Ching Research Fund. We thank Dr. Tim Yandle (Christchurch Hospital, Christchurch, New Zealand) for advice on the assay of AngII.

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