Precocious activation of genes of the renin-angiotensin system and the fibrogenic cascade in IgA glomerulonephritis

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Precocious activation of genes of the renin-angiotensin system and the fibrogenic cascade in IgA glomerulonephritis.

Background. The renin-angiotensin system (RAS) seems to play a pivotal role in progression of immunoglobulin A (IgA) nephropathy (IgAN). Accordingly, in patients with IgAN a relationship between the RAS and the fibrogenic cascade triggered by transforming growth factor-β1 (TGF-β1) should be observed. This study was carried out to obtain deeper insight into the regulation of RAS and the interaction with TGF-β1 in the diseased kidney.

Methods. Twenty renal biopsies from IgAN patients and five from renal cancer patients (controls) were analyzed in both microdissected glomerular and tubulointerstitial compartments by reverse transcription-polymerase chain reaction (RT-PCR). All patients had normal renal function. The expression of the following genes was determined: angiotensinogen (*Agtg*), renin, angiotensin-converting enzyme (*ACE*), angiotensin II (Ang II) type 1 and type II (*AT1* and *AT2 receptors*), *TGF-β1*, collagen IV (*Coll IV*), α-smooth muscle actin (*α-SMA*). Quantitative data were confirmed for *TGF-β1* and *ACE* genes by real-time PCR.

Results. RAS genes were overexpressed in IgAN patients vs. control subjects. There was no difference between glomerular and tubulointerstitial *RAS* gene expression levels. On the contrary, the overactivation of fibrogenic cascade genes (*TGF-β1, Coll IV, α-SMA*) in the tubulointerstitium was observed (*TGF-β1*, glomerular 0.14 ± 0.10 SD; tubulointerstial 0.34 ± 0.20; $P = 0.000$) (*α-SMA*, glomerular 0.08 ± 0.07; tubulointerstitial 0.35 ± 0.19; $P = 0.000$) (*Coll IV*, glomerular 0.12 ± 0.11; tubulointerstitial 0.22 ± 0.10; $P = 0.03$). This fibrogenic cascade seems to be triggered by RAS as indicated by statistically significant correlations between the expression of their respective genes. A direct relationship between the putative Ang II activity and the expression of *AT receptor* genes was found in the tubulointerstitium, whereas in the glomeruli this relationship was negative. In the interstitium, statistically significant positive relationships emerged between interstitial infiltrates and the gene expression of *Agtg*, *AT1 receptor*, *Coll IV*, and *TGF-β1*.

Conclusion. This study demonstrates that a tight regulation of the intrarenal RAS exists in IgAN and that it follows the general rules disclosed in animal models. Moreover, the RAS seems to be activated early in the diseased kidney and it appears that such activation drives inflammation and a parallel stimulation of the TGF-β fibrogenic loop, particularly at the tubulointerstitial level.

Angiotensin II (Ang II), an octapeptide hormone that is the major effector molecule of the renin-angiotensin system (RAS), acts as a circulating hormone as well as in a paracrine and/or an autocrine fashion to modulate renal function. Indeed, a number of studies have shown that all components of the RAS are widely distributed in human tissues and specifically in the kidney. Ang II has been shown to increase efferent arteriolar resistance, glomerular capillary hydraulic pressure and to decrease plasma flow rate, glomerular filtration rate (GFR), ultrafiltration coefficient, and hydraulic conductivity in the glomerulus [1]. It has also been implicated in the autoregulation of renal blood flow and many studies have shown that Ang II plays an important role in regulating the tubuloglomerular feedback [2, 3]. Furthermore, a growing body of evidence supports the notion that Ang II may play a central role in the pathophysiology of renal diseases in humans. Ang II, via type 1 Ang II (AT1) receptor, directly causes cellular phenotypic changes and cell growth, regulates the gene expression of various bioactive substances (vasoactive hormones, growth factors, extracellular matrix components, cytokines, etc.), and activates multiple intracellular signaling cascades (mitogen-acti-

vated protein kinase cascades, tyrosine kinases, various transcription factors, etc.) in fibroblasts, endothelial, and renal mesangial cells. These actions are supposed to participate in the pathophysiology of glomerulosclerosis [4].

Surprisingly, little information is available about the RAS regulation in the human kidney and particularly in kidney diseases and data on the *RAS* gene expression and regulation were mostly obtained in animals [5–8]. These data in humans and in diseased kidneys would be worthwhile evaluating because changes in plasma RAS do not closely reflect local expression and regulation of the renal RAS [9–14]. On the contrary, the simultaneous assessment of the intrarenal expression and regulation of all components of the RAS is necessary for evaluation of the net effect of the RAS on the kidney. Indeed, the effect of the activity of the RAS on the kidney cannot be accurately assessed by the measurement of one component alone. For instance, the local availability and functional consequence of Ang II, the effector of the system, may depend, among others, on the angiotensin-converting enzyme (ACE) concentration or on the Ang II receptor density. However, available data, and namely the human data, are fragmentary since only few RAS components have been evaluated, and generally not more than one at a time.

This study was carried out to obtain deeper insight on the RAS regulation in the diseased kidney in view of the pathogenic role of this system, particularly in the progression to renal failure [15, 16]. As a paradigmatic renal disorder we investigated immunoglobulin A (IgA) nephropathy (IgAN), the most common type of primary glomerulonephritis worldwide and a major cause of end stage renal failure (ESRF) [17, 18]. All known components of the RAS and some of the transforming growth factor-β (TGF-β) axis were simultaneously investigated in both the glomerular and tubulointerstitial compartments by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) approach set up in our laboratory [19].

METHODS

Forty patients with a clinical and laboratory pattern suggesting IgAN (recurrent bouts of macrohematuria or persistent microhematuria associated with proteinuria) (age range, 20 to 42 years, 29 males) and eight control patients (35 to 54 years, 6 males) constitute the basis of this study. All gave informed, written consent.

Patients were consecutively enrolled in the participating units of the study. From January 1999 to October 2000, in patients who were selected for renal biopsy, a sodium-controlled diet (5 g per day) was administered the week before biopsy. Diet compliance was checked by evaluating the urinary excretion of sodium the day before biopsy and a renal specimen fragment was immediately microdissected under stereomicroscopic examination as outlined below. RNA was extracted from both
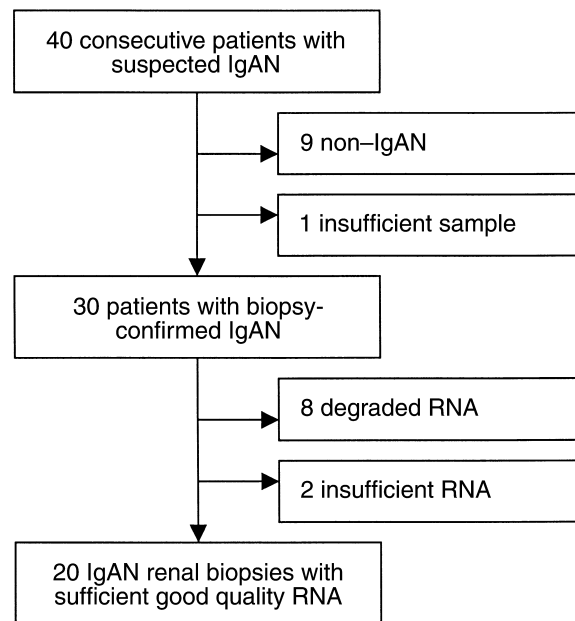

Fig. 1. Disposition of patients and samples. IgAN is immunoglobulin A nephropathy.

compartments on the same day and stored at −80°C until further use. At enrollment, patients who had been treated for hypertension were placed in pharmacologic washout for at least 10 days for ACE inhibitors, AT1 receptor inhibitors, beta blockers, clonidine, and diuretics. Exclusion criteria from the study were age below 18 years, and over 65 years; non-Caucasian; serum creatinine exceeding 180 μmol/L; nephrotic range proteinuria; treatment with steroids, nonsteroidal anti-inflammatory drugs (NSAIDs) or immunosuppressants; pregnancy or contraceptive use in the month before biopsy; diabetes mellitus; renovascular hypertension; obstructive uropathy; neoplasia; liver disease; alcohol or drug abuse; connective tissue diseases; Henoch-Schonlein purpura; familial IgAN; or other contraindications for performing a renal biopsy.

Renal biopsies were obtained under ultrasound guidance with a 14-gauge needle. Only those biopsies disclosing the typical immunofluorescence for IgAN and providing a sufficient sample for performing both the standard pathologic examination and molecular biology analysis (30 out of 40), were considered for this study (Fig. 1).

Light microscopy and immunofluorescence were also performed on renal tissue surgical specimens, obtained with a 14-gauge needle before renal artery clamping from renal cancer patients undergoing nephrectomy (controls), after a 1-week 5 g sodium-controlled diet. Specimens were taken from sites remote from tumor-bearing tissue. Only those biopsies disclosing a normal morphology and a negative immunofluorescence (five out eight) were considered. Renal specimens were immediately microdissected and RNA extracted.

Microdissection and RNA extraction

In all samples (IgAN and controls) immediately after biopsy, approximately one tenth of the specimen was kept in a physiologic solution containing 100 U of RNAsin (Perkin Elmer, Branchburg, NJ, USA) on ice. Three to 21 glomeruli and corresponding tubulointerstitium were isolated under microscopic control, using a stereomicroscope (Zeiss, Jena, Germany), and immediately put into RNAzolB solution (BIOTEX, Houston, TX, USA). Total RNA was extracted using the RNA-zolB method with some minor modifications. Glomeruli and tubulointerstitium were homogenized in 200 μL of RNAzolB solution by pipetting. The homogenate was added with 0.1 volume of chloroform, then shaken vigorously, kept on ice for 5 minutes and centrifuged at 14,000*g* (4°C) for 20 minutes. The aqueous phase was transferred to a fresh tube, added to an equal volume of isopropanol, stored for 45 minutes on ice and centrifuged for 20 minutes at 14,000*g* (4°C). The supernatant was removed and the RNA pellet was washed with 70% ethanol. To obtain a purer RNA preparation for the subsequent enzymatic assay, an additional overnight precipitation step with 2 volumes of 100% ethanol at −20°C was performed. The RNA pellet was dissolved in 10 μL of diethyl pyrocarbonate (DEPC) water. Five microliters of RNA were used for the spectrophotometric quantitation at 260 and 280 nm using a 50 μL microcell (Perkin Elmer). Amplifying the 983 bp fragment of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) checked RNA integrity [19].

Reverse transcription (RT)

Thirty nanograms of total RNA were retrotranscribed in a final volume of 20 μL, in the presence of a 5 mmol/L MgCl$_2$, 1 mmol/L desoxynucleoside triphosphate (dNTP), 1 U/μL RNAse inhibitor, 2.5 U/μL MuLV Reverse Transcriptase (Perkin Elmer), 2.5 μmol/L Random Examers in buffer 50 mmol/L KCl, 10 mmol/L Tris HCl, pH 8.3. This reaction was carried out for 30 minutes at 42°C, and 5 minutes at 99°C in a thermalcycler (M.J. Research, Inc., Waltham, MA, USA).

Polymerase chain reaction (PCR)

An aliquot of 1 μL of RT reaction was used to amplify in different tubes the following genes: *Agtg*, *renin*, *ACE*, *AT1 receptor*, type 2 Ang II (*AT2 receptor*), *TGF-β1*, α1 chain of collagen IV (*Coll IV*), α-smooth muscle actin (*α-SMA*) and the housekeeping gene *G3PDH* as internal standard. The specific cDNA sequences were amplified using the following primers:

Agtg
forward, CTGCAAGGATCTTATGACCTGC and reverse, TACACAGCAAACAGGAATGGGC, amplification product of 217 bp [20];

renin
forward, AAATGAAGGGGGTGTCTGTGG and reverse, AAGCCAATGCGGTTGTTACGC, amplification product of 376 bp [20];

ACE
forward, GCCTCCCCAACAAGACTGCCA and reverse, CCACATGTCTCCAGCCAGATG, amplification product of 388 bp [20];

AT1 receptor
forward, GGCCAGTGTTTTTCTTTTGAATTT and reverse, TGAACAATAGCCAGGTATCGATCAATG, amplification product of 210 bp;

AT2 receptor
forward, GTGGCTGATTTACTCCTTTTGG and reverse, TATAAGATGCTTGCCAGGGATT, amplification product of 226 bp;

TGF-β1
forward, GCCCTGGACACCAACTATTGCT and reverse, AGGCTCCAAATGTAGGGGCAGG, amplification product of 162 bp (Clontech, Palo Alto, CA, USA);

Coll IV
forward, TTTGCATCACGAAATGACTAC and reverse, AAGGTGGACGGCGTAGGCTTC, amplification product of 413 bp [21];

α-SMA
forward, CTGCCTTGGTGTGTGACAAT and reverse, ATTGTGGGTGACACCATCTC, amplification product of 470 bp;

G3PDH
forward, ACCACAGTCCATGCCATCAC and reverse, TCCACCACCCTGTTGCTGTA, amplification product of 452 bp (Clontech).

Although the primers were designed to span one or more introns within the genes, control negative reactions, without reverse transcriptase, were performed during the cDNA synthesis step in order to exclude genomic contamination. To increase the specificity and the efficiency of the PCR reaction, the "hot start" procedure was applied by the use of a Jump Start Taq (Sigma Chemical Co., St. Louis, MO, USA). The amplification was carried out in a final volume of 50 μL containing 1.5 mmol/L MgCl$_2$, 0.2 mmol/L dNTP, 1 U Jump Start Taq (Sigma Chemical Co.), and 0.4 μmol/L primers in 50 mmol/L KCl and 10 mmol/L Tris HCl, pH 8. cDNAs

were amplified according to the following conditions: 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes.

Comparative RT-PCR kinetic analysis

Kinetic analysis of amplified products was applied to all samples for each genes to ensure that signals were derived only from the exponential phase of the amplification. cDNAs were submitted to the first 30 cycles of amplification, and an aliquot of 5 μL from each sample was drawn for electrophoretic analysis. Then the tubes were submitted to two more cycles of amplification and one more 5 μL aliquot was drawn. This procedure was repeated six times until it reached a total of 40 cycles. PCR products obtained after 30, 32, 34, 36, 38, and 40 cycles were analyzed by polyacrylamide gel electrophoresis (PAGE) in 7% polyacrylamide gel, 3% C with 5% glycerol at 150 constant voltage in $1 \times$ Tris-borate-EDTA buffer (TBE) for $1\frac{1}{2}$ hours, visualized by ethidium bromide staining and photographed. To enhance band signals, gels were silver stained according to the following protocol: 10% ethanol for 5 minutes, 1% HNO_3 for 3 minutes, rinsed in distilled water, 12 mmol/L $AgNO_3$ for 20 minutes, rinsed in distilled water, and developed in 280 mmol/L $NaCO_3$ and 0.019% formaldehyde until the desired staining was reached; the development was stopped with 10% CH_3COOH for 2 minutes.

After determining for each gene in each sample the exponential phase of reaction by kinetic PCR, we selected the appropriate cycles in which PCR products had been quantified: *G3PDH*, 32 cycles; *Agtg*, 34 cycles; *renin*, 34 cycles; *ACE*, 38 cycles; *AT1 receptor*, 36 cycles; *AT2 receptor*, 38 cycles; *TGF-β1*, 32 cycles; *Coll IV*, 32 cycles; and *α-SMA*, 34 cycles. The quantification of PCR products was performed by direct densitometric analysis of silver-stained bands using Gel-Pro Analyser software (Media Cybernetics, Silver Springs, MD, USA) and the quantity of the different mRNAs was expressed as the ratio between optical density (OD) generated by PCR products of the different genes and the *G3PDH* gene.

Real-time PCR quantification using SYBR Green I

Few genes (*ACE*, *TGF-β*, and *G3PDH*) were also evaluated by real-time PCR. Real-time PCR was performed using the iCycler Thermal Cycler (BioRad, Hercules, CA, USA) and the SYBR Green I analysis. The PCR standard for *ACE*, *TGF-β1*, and *G3PDH* consisted of known numbers of molecules of purified PCR products. After checking the specificity by PAGE analysis, PCR products were purified using MinElute PCR Purification Kit (Qiagen, Bothell, WA, USA), and quantified by spectrophotometry at 260 nm wavelength. The number of copies/mL standard was calculated according to the following formula:

$$copies/mL = \frac{6.023 \times 10^{23} \times C \times OD_{260}}{MWt}$$

where $C = 5 \times 10^{-5}$ g/mL for DNA and MWt = molecular weight of PCR product gene (base pairs $\times$ 6.58 $\times$ 10^2 g). Standards were serially diluted in log steps from 10^8 down to 10 copies in 1 μL volume. The sensitivity of the PCR method using different primers was determined from the threshold cycle values obtained with known quantities of purified PCR products. All calibration curves for purified PCR products, *ACE*, *TGF-β1*, and *G3PDH* showed linearity over the entire quantification range with correlation coefficients $r = 0.98$, indicating a precise log-linear relationship. The slopes of three genes were 3.5, 3.59, and 3.6, respectively, demonstrating comparable PCR amplification efficiencies. The intrarun variability, calculated from duplicate samples for all the targets, showed an average SD for the threshold cycles of 0.12 cycles. The primers employed to amplify using SYBR Green I were, respectively, for *G3PDH*, forward 5′-GAAGGTGAAGGTCGGAGT-3′, reverse 5′-TGG CAACAATATCCACTTTACCA-3′; for *TGF-β1*, forward 5′-TTATCTTTTGATGTCACCGGAGT-3′, reverse 5′-GTAGTGAACCCGTTGATGTC-3′; and for *ACE*, forward 5′-ATGAAGACCTGTTATGGGCA TGG-3′, reverse 5′-ATTTCGGGTAAAACTGGAG GATGG-3′.

The size of PCR products amplified with primers for SYBR Green I analysis were *G3PDH*, 92 bp; *ACE*, 75 bp; and *TGF-β*, 137 bp. The real-time PCR quantification was performed starting from the same RT reaction of semiquantitative RT-PCR, and the same cDNA (1 μL) quantity.

The optimal concentration of primers (300 nmol/L) and $MgCl_2$ (3 mmol/L) was determined in preliminary experiments. The thermal cycling profile for *G3PDH*, *TGF-β1*, and *ACE* consisted of: step 1, 95°C for 5 minutes; step 2, 94°C for 45 seconds; step 3, 60°C for 30 seconds (steps 2 and 3 repeated for 40 cycles); and step 4, melting curve. As SYBR Green I also binds to primer dimers formed nonspecifically during all PCR reactions, it was necessary to obtain the most favorable temperature for analysis of specific product. Melting curve analysis and PAGE confirmed the specificity of the amplification products. The quantification data were analyzed with iCycler analysis software and expressed as the ratio between starting quantity mean (SQm) of target and housekeeping gene.

Histopathologic evaluation

One of the investigators reviewed all the histologic specimens and for each biopsy three serial sections stained with hematoxylin-eosin, periodic-acid Schiff (PAS), and Masson's trichrome were evaluated. The overall severity of renal damage was graded according to the Lee's classi-

Table 1. Clinical, histopathologic and demographic characteristics of subjects

	Patients	Controls
Gender *M/F*	29/11	6/2
Age *years*	30 (20–42)	42 (35–54)
Proteinuria *g/24 hours*	1.3 (0.9–2.0)	/
Hypertension *yes/no*	11/29	2/6
Serum Creatinine *μmol/L*	98 (79–119)	103 (85–131)
Global glomerular sclerosis %	5 (0–67)	/
Diffuse mesangial proliferation %	12 (0–78)	/
Crescents %	0 (0–18)	/
Interstitial infiltrates	0 (0–2)	/
Interstitial fibrosis	0 (0–2)	/
Vascular lesions	0 (0–1)	/
Lee's score	3 (2–4)	/

Values are median and (ranges) or absolute numbers (i.e., number of subjects).

fication [22]: grades I and II = mild renal damage, grade III = moderate renal damage, and grade IV and V = severe renal damage. Moreover, in each biopsy six features were assessed: global glomerular sclerosis, crescents, diffuse mesangial proliferation, interstitial fibrosis, interstitial infiltrates, and vascular lesions. Global glomerular sclerosis was expressed as percentage of glomeruli with global sclerosis over the total number of glomeruli in each biopsy. Diffuse mesangial proliferation and crescents were evaluated as percentage of glomeruli interested by the lesions over the number of glomeruli without global sclerosis. Interstitial fibrosis and infiltrates were graded as moderate (if absent or focal) or severe (if multifocal or diffuse). Vascular lesions (arteriolar hyalinosis and arteriosclerosis) were considered as present or absent.

Statistics

Statistics were carried out by linear regression analysis of the different gene expression levels. The Student *t* test was used to compare mRNA levels between glomeruli and tubulointerstitium. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical, histopathologic, and demographic characteristics of enrolled patients are shown in Table 1. Because of RNA degradation (checked by evaluating the integrity of the 983 bp *G3PDH* amplification product) [19] and poor total RNA yield (less than 30 ng of RNA recovered from renal biopsy), 10 out of 30 IgAN biopsies were excluded from the analysis. Thus, 20 biopsies were considered (Fig. 1). The expression levels of RAS components and of the TGF-β axis in two kidney compartments (glomeruli and tubulointerstitium) were evaluated by RT-PCR. However, to confirm the reliability of this procedure as a quantitative tool, the expression of three index genes (*TGF-β1*, *ACE*, and the housekeeper
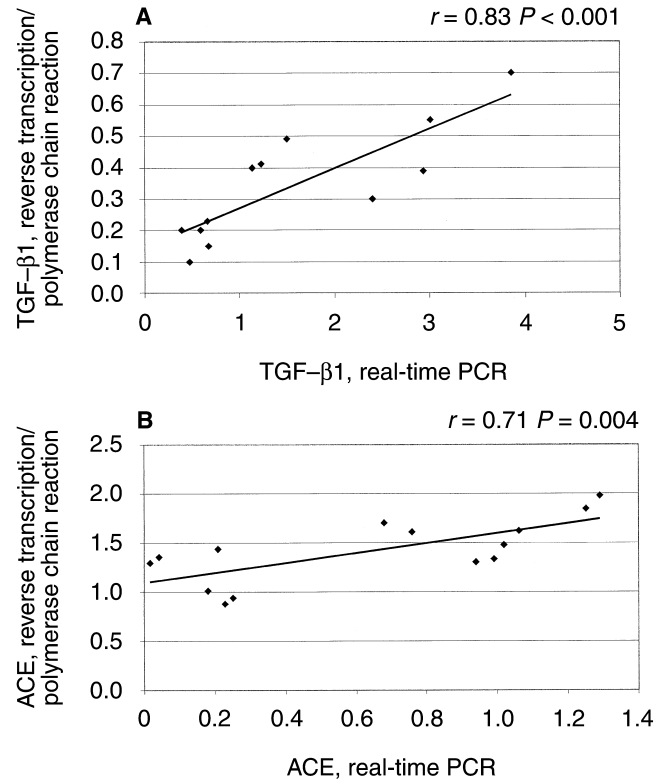

Fig. 2. Relationship between real-time polymerase chain reaction (PCR) and reverse transcription (RT)-PCR values of transforming growth factor-β1 (*TGF-β1*) (*A*) and angiotensin-converting enzyme (*ACE*) (*B*) genes expression. Insufficient RNA precluded real-time PCR analysis in some specimens. For this reason, only data points of 12 (A) and 14 (B) patients instead of 20 are shown. In the x-axes, the ratio between number of copies of *TGF-β1* or *ACE*, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) determined by real-time PCR is given. In the y-axes, the ratio between optical densities of *TGF-β1*, *ACE*, and *G3PDH* determined by RT-PCR is shown.

G3PDH) was also evaluated by real-time PCR. A statistically significant direct correlation was demonstrated between RT and real-time PCR values for *TGF-β1* and *ACE* ($r = 0.83$ $P < 0.001$; $r = 0.71$ $P = 0.004$, respectively) (Fig. 2).

In IgAN patients, we found no difference between glomerular and tubulointerstitial *RAS* gene expression levels, while the expression levels of *TGF-β1*, *Coll IV*, and *a-SMA* genes were significantly higher in the tubulointerstitium (*TGF-β1*, glomerular 0.14 ± 0.10 SD; tubulointerstitial 0.34 ± 0.20; $P < 0.001$; *a-SMA*, glomerular 0.08 ± 0.07; tubulointerstitial 0.35 ± 0.19; $P < 0.001$; *Coll IV*, glomerular 0.12 ± 0.11; tubulointerstitial 0.22 ± 0.10; $P = 0.03$) as shown in Figure 3. Molecular analysis performed in control biopsies revealed almost undetectable expression levels of *RAS* genes and in particular *Agtg* and *ACE* genes were not expressed (Fig. 4).

Table 2 reports the results of correlations between RAS components in glomeruli and tubulointerstitium in IgAN patients. In glomeruli, *Agtg* mRNA was inversely
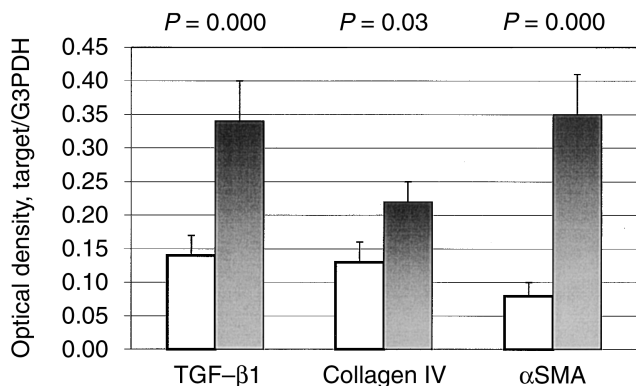
Fig. 3. Expression of fibrogenic cascade genes in renal biopsies from immunoglobulin A nephropathy (IgAN) patient. The relative quantitation between optical densities of the target gene [transforming growth factor-β1 (*TGF-β1*), collagen IV (*Coll IV*), α-smooth muscle actin (*a-SMA*) and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene is given in the glomerular (□) and tubulointerstitial (■) compartments.

correlated with both *AT1 receptor* and *AT2 receptor* gene expression ($r = -059$; $P < 0.01$ and $r = -0.64$; $P < 0.005$, respectively), and positively with *renin* ($r = 0.60$; $P < 0.009$), with *ACE* ($r = 0.61$; $P < 0.008$) and with *TGF-β1* ($r = 0.70$; $P < 0.001$). A positive correlation emerged between mRNA levels of *renin* and *ACE* ($r = 0.61$; $P < 0.008$), and *renin* and *TGF-β1* ($r = 0.47$; $P < 0.05$); on the contrary, *renin* and *AT2 receptors* were linked through an inverse relationship ($r = -0.46$; $P < 0.05$). *ACE* gene expression was negatively correlated with *AT1 receptor* and *AT2 receptor* expression ($r = -0.47$; $P < 0.05$; $r = -0.59$; $P < 0.01$, respectively), positively with *TGF-β1* ($r = 0.51$; $P < 0.03$) and with *Coll IV* ($r = 0.59$; $P < 0.01$). *AT1 receptor* mRNA level was positively correlated with *AT2 receptor* mRNA levels ($r = 0.68$; $P < 0.002$), but negatively correlated to *a-SMA* ($r = -0.53$; $P < 0.03$), *TGF-β1* ($r = -0.52$; $P < 0.02$) and with *Coll IV* ($r = -0.53$; $P < 0.03$). *AT2 receptor* expression was inversely correlated with *TGF-β1* ($r = -0.51$; $P < 0.03$). Finally, *TGF-β1* was positively correlated with *Coll IV* ($r = 0.85$; $P < 0.001$) and with *a-SMA* ($r = 0.46$; $P < 0.05$). No other correlation was evidenced between the expression of explored genes in glomeruli. A schematic representation is shown in Figure 5.

In the tubulointerstitial compartment, *Agtg* mRNA levels were positively correlated with the gene expression of *ACE* ($r = 0.87$; $P < 0.001$), with *AT1 receptor* ($r = 0.66$; $P < 0.02$), with *a-SMA* ($r = 0.64$; $P < 0.03$) and *Coll IV* ($r = 0.71$; $P < 0.01$). *ACE* gene expression was positively correlated with *a-SMA* gene expression ($r = 0.90$; $P < 0.001$). *AT1 receptors* and *AT2 receptors* were both positively correlated with *TGF-β1* ($r = 0.64$; $P < 0.03$; and $r = 0.69$; $P < 0.01$, respectively). Finally, a positive correlation was demonstrated between *TGF-β1* and *Coll IV* ($r = 0.63$; $P < 0.03$). No other correlation

was evidenced between the expression of explored genes in the tubulointerstitial compartment. A schematic representation is shown in Figure 6.

There was no statistically significant correlation between glomerular histopathologic features and mRNA glomerular levels of *RAS* and fibrogenic genes. On the contrary, in the interstitium, statistically significant positive relationships emerged between interstitial infiltrates and the interstitial gene expression of *Agtg* ($r = 0.61$; $P < 0.008$), *AT1 receptor* ($r = 0.84$; $P < 0.001$), *Coll IV* ($r = 0.71$; $P < 0.01$), *TGF-β1* ($r = 0.71$; $P < 0.01$); the Lee's score was also positively correlated with the interstitial *AT1 receptor* gene levels ($r = 0.65$; $P < 0.03$).

DISCUSSION

This study suggests that intrarenal RAS in human IgAN is overexpressed and is strictly regulated as demonstrated by the correlation between different components of the RAS cascade. It also shows that the fibrogenic cascade is distinctively overexpressed in the tubulointerstitial compartment and with the overactivated RAS seem to incite renal lesions.

The aim of this study was to obtain a comprehensive evaluation of RAS in glomeruli and tubulointerstitium of patients with IgAN to overcome the many limits of the few available human investigations. This goal was addressed by evaluating simultaneously in the same biopsy, the maximum number of components of this system and of the fibrogenic cascade functionally linked to it, by a semiquantitative comparative kinetic RT-PCR approach developed in our laboratory [19]. We verified the reliability of RT-PCR results through the quantification of some of the gene expressions with the real-time PCR technology. There was a statistically significant correlation for both *ACE* and *TGF-β1* between semiquantitative RT and real-time PCR values (Fig. 2), supporting the reliability of our data and analysis.

The molecular biology approach addressing the determination of gene expression of the RAS and fibrogenic cascade components in the kidney does not allow the determination of activity nor the regulation of either Ang I or Ang II. However, the latter can be inferred from the relationship between the expression of upstream (hereafter, the terms "upstream" or "upward" will be used to indicate RAS components preceding Ang II; the contrary for the components following Ang II in the RAS cascade) enzyme genes, *ACE* and *renin*, and the substrate *Agtg* gene. Furthermore, mRNA levels alone may not accurately reflect the overall level of expression of the RAS protein components and of Ang II–mediated events. RNA stability, posttranslational processing, changes in receptor cycling, and second-messenger uncoupling of AT receptors may modulate signal transduction. However, at least for some RAS compo-
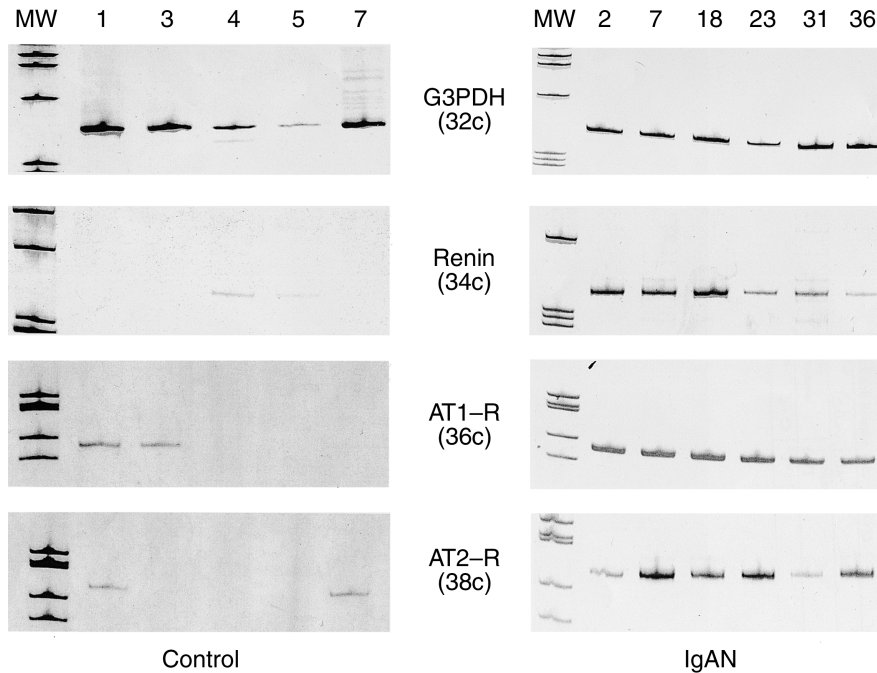
Fig. 4. Expression of renin-angiotensin system (RAS) genes in controls and immunoglobulin A nephropathy (IgAN) patient renal biopsies. Silver-stained gels of polymerase chain reaction (PCR) products are shown. The expression of some *RAS* genes in glomeruli from few controls and IgAN patients is reported as an example. Controls and IgAN patients are indicated by their respective enrollment number. RNA levels were very faint in controls vs. IgAN patient renal biopsies. Angiotensinogen (*Agtg*) and angiotensin-converting enzyme (*ACE*) genes were not expressed in controls. Number of amplification cycles (c) are reported in brackets. Abbreviations are: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight; AT1-R, angiotensin II type 1 receptor; AT2-R, angiotensin II type 2 receptor.

nents, a reasonably close relationship between mRNA levels, on the one hand, and Ang II binding, on the other, were reported [23, 24].

Due to the key role of *renin*, the rate-limiting step in the classic RAS cascade [25], it seems logical to observe direct correlations between *renin* and *Agtg*, and *renin* and *ACE* in the glomerulus (Fig. 5). These direct relationships suggest that parallel, proportional modifications occur in the intraglomerular activity of Ang I and Ang II. The inverse relationships between AT receptors and upward RAS components (i.e., *ACE*, *renin*, and *Agtg* also point to the same conclusion). It has been reported that a negative feedback exists between Ang II and its type 1 receptors in glomerular cells [23, 24]. Therefore, if the upstream-positive correlations suggest a proportional activation of Ang II, it is coherent to expect a proportional deactivation of the AT receptors (i.e., a negative relationship between AT receptors and the upstream RAS components).

Interestingly, the expression of the two AT receptor genes is strictly correlated. Indeed, a recent body of data supports the concept that AT2 receptor counterbalances the activity and the effects of AT1 receptor, disclosing opposing features in many aspects of their biologic function, and particularly with respect to the intrarenal RAS, in cell growth and proliferation [26–28]. This suggests a tight functional link between the two receptors, a concept, which is supported by the positive correlation observed in this study (Table 2, Fig. 5).

The components of the fibrogenic loop (i.e., *TGF-β1*, *Coll IV*, and *α-SMA*) are all or in part positively corre-

lated with some of the upstream components of the RAS (i.e., *Agtg* and *ACE*); on the other hand, they are inversely correlated to the *AT1 receptor* expression. Again this is a logical expectation since (*1*) as previously discussed, the positive correlations between the upstream RAS components, on one hand, and the negative correlations between these components with the downstream AT receptors, on the other hand, support the concept of a concord intrarenal activity of Ang II; (*2*) Ang II is believed to trigger the synthesis of *TGF-β1*, and some of their activities are indeed induced through the mediation of this cytokine [29].

While the inverse correlation between *AT2 receptor* and *ACE* confirms previous findings in the knockout model where the AT2 receptor was shown to inhibit ACE [30], the inverse relationship between the AT2 receptor expression and *TGF-β1* is puzzling. Indeed, it is not supported by any literature, which, on the contrary, suggests that these receptors do not modulate the *TGF-β* release [31] and offers a quite different, antifibrogenic outcome after AT2 receptor stimulation [27, 32]. Most likely it is a spurious correlation due to the much stronger association between *AT1 receptor* with *TGF-β1*, and *AT1 receptor* with *AT2 receptor*.

Findings in the tubulointerstitium substantially confirm and support the data observed in glomeruli, with just one very interesting exception [i.e., the direct correlation existing between *Agtg* (and, because of the above considerations, we infer with Ang II activity) with *AT1 receptor*]. As a matter of fact, in the rat it was shown that Ang II up-regulates AT1 receptors in tubular cells [33],

Table 2. Correlations between renin-angiotensin system (RAS) components in glomeruli and tubulointerstitium in immunoglobulin A nephropathy (IgAN) patients

	Glomeruli		Tubulointerstitium	
	r value	P value	r value	P value
ACE/renin	+0.61	<0.008		
ACE/AT1-R	−0.47	<0.05		
ACE/AT2-R	−0.59	<0.01		
ACE/TGF-β1	+0.51	<0.03		
ACE/Coll IV	+0.59	<0.01		
ACE/α-SMA			+0.90	<0.001
Renin/AT2-R	−0.46	<0.05		
Renin/TGF-β1	+0.47	<0.05		
Agtg/renin	+0.60	<0.009		
Agtg/ACE	+0.61	<0.008	+0.87	<0.001
Agtg/AT1-R	−0.59	<0.01	+0.66	<0.02
Agtg/AT2-R	−0.64	<0.005		
Agtg/TGF-β1	+0.70	<0.001		
Agtg/α-SMA			+0.64	<0.03
Agtg/Coll IV			+0.71	<0.01
AT1-R/AT2-R	+0.68	<0.002		
AT1-R/TGF-β1	−0.52	<0.02	+0.64	<0.03
AT1-R/α-SMA	−0.53	<0.03		
AT1-R/Coll IV	−0.53	<0.03		
AT2-R/TGF-β1	−0.51	<0.03	+0.69	<0.01
TGF-β1/Coll IV	+0.85	<0.001	+0.63	<0.03
TGF-β1/α-SMA	+0.46	<0.05		

Abbreviations are: *Agtg*, angiotensinogen; *ACE*, angiotensin-converting enzyme; *AT1-R*, angiotensin II type 1 receptor; *AT2-R*, angiotensin II type 2 receptor; *α-SMA*, α-smooth muscle actin; *TGF-β*, transforming growth factor-β; *Coll IV*, collagen IV.
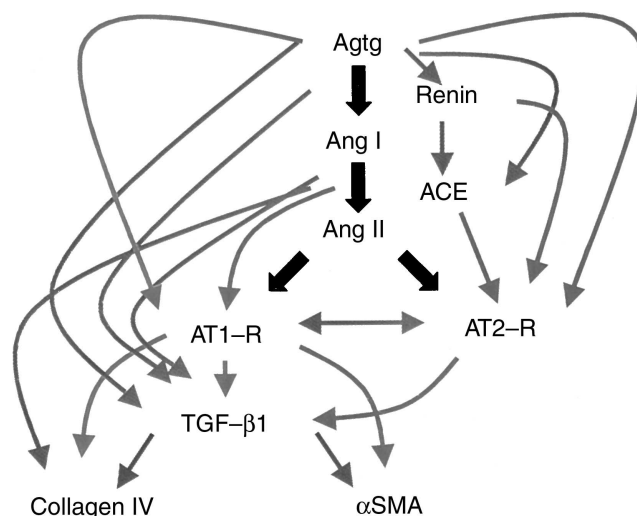

Fig. 5. Regulation of the renin-angiotensin system (RAS) and of the transforming growth factor-β (TGF-β) loops in glomeruli of immunoglobulin A nephropathy (IgAN) patients. Molecules whose levels were not determined are underlined; dotted lines indicate a statistically significant negative relationship, while solid lines express positive correlations. Empty arrows indicate relationships that were not investigated. Abbreviations are: Agtg, angiotensinogen; Ang I, angiotensin I, Ang II, angiotensin II, ACE, angiotensin-converting enzyme; AT1-R, angiotensin II type 1 receptor; AT2-R, angiotensin II type 2 receptor; α-SMA, α-smooth muscle actin.

contrarily to what happens in glomerular cells [23, 24]. There is no data on such a different regulation in the human kidney. The functional meaning of this opposite control is unknown; it may well deal with an integrated activity of the RAS between the tubule and the glomerulus rather than to some distinct and autonomous effect in the two compartments. For instance, Cheng et al [33] suggested that the regulation of proximal tubule AT1 receptors by Ang II may be important in modulating sodium reabsorption in the proximal tubule according to the systemic or intrarenal Ang II levels (i.e., depending on the status of fluid volumes). In the glomerulus, the different regulation between Ang II and AT1 receptors could be relevant to the resetting of the tubuloglomerular feedback (i.e., the attenuation in tubuloglomerular feedback activity that contributes to increased natriuresis and diuresis during expansion of extracellular fluid volume and thus facilitates return of blood volume to the initial euvolemic set point [34]).

We speculate that such an opposite regulation in the RAS in the tubule versus the glomerulus due to a hierarchical superior function (control of euvolemia) may have some undue consequences in pathologic conditions because of additional Ang II activities, namely the proinflammatory and fibrogenic ones. With reference to this point, the positive correlation in the tubulointerstitial compartment most likely corresponds to a positive feed-back that translates in the enrollment of inflammatory cells, and in the overactivation of the fibrogenic cascade. In glomeruli, the situation can be quite different since the fibrogenic balance looks more strictly controlled because of the inverse relationship between Ang II and AT1 receptor levels. Two order of findings in the tubulointerstitial compartment support this view: (*1*) the expression levels of *TGF-β1*, *Coll IV*, and *α-SMA* genes are all significantly higher than in the glomerulus (Fig. 3); (*2*) a positive relationship between the histopathologic feature of "interstitial infiltrates" and *Agtg*, *AT1 receptor*, *Coll IV*, and *TGF-β1* mRNAs can be observed at odds with the glomerular level. These findings in the tubulointerstitium probably reflect the mechanisms responsible for the unfavorable evolution of renal disease. Indeed, one of the most robust predictors of poor prognosis in human nephropathies, including IgAN, is interstitial fibrosis and inflammation rather than glomerular damage [35].

The relationship between *TGF-β1* and *RAS* gene tubulointerstitial expression, and inflammatory infiltrates in IgAN patients should not be surprising. Actually Ang II increases monocyte adhesion to the endothelium [36] and is chemotactic for neutrophil leukocyte [37] and monocyte/macrophage [38, 39] and a role of the RAS in renal inflammation has been proposed [40]. TGF-β1 is also chemotactic for monocytes [41]. Although we cannot rule out the possibility that this relationship only reflects the presence in the interstitium of inflammatory
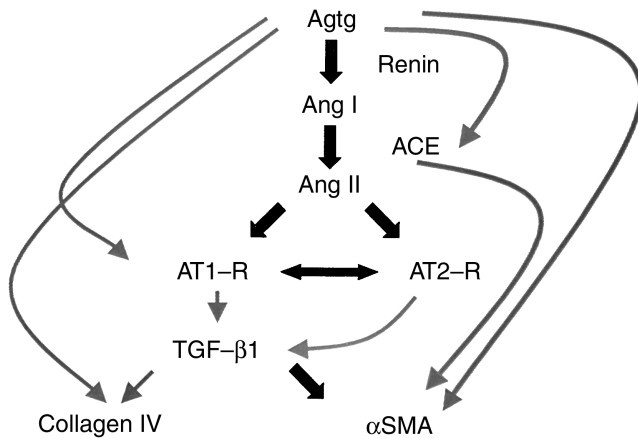
Fig. 6. Regulation of the renin-angiotensin system (RAS) and of the transforming growth factor-β (TGF-β) loops in the tubulointerstitial compartment of immunoglobulin A nephropathy (IgAN) patients. Molecules whose levels were not determined are underlined; solid lines express positive correlations. Empty arrows indicate relationships that were not investigated. Abbreviations are: Agtg, angiotensinogen; Ang I, angiotensin I; Ang II, angiotensin II; ACE, angiotensin-converting enzyme; AT1-R, angiotensin II type 1 receptor; AT2-R, angiotensin II type 2 receptor; α-SMA, α-smooth muscle actin.

cells since these cells also express genes of the RAS [42, 43], the lack of such a relationship in glomeruli [44], and the in situ hybridization study by Lai et al [45] that did not report *RAS* gene expression in inflammatory cells in IgAN support the idea that, at least in reference to the RAS, interstitial infiltrates could be a phenomenon secondary to RAS activation. Lack of correlation between interstitial fibrosis and RAS or fibrogenic cascade genes is probably explained by the fact that we investigated the early stages of IgAN as demonstrated by the modest severity of renal histopathologic characteristics in our patients (Table 1). Thus, we most likely explored a stage of the disease in which interstitial inflammation precedes fibrosis and sclerosis. This, however, can be foreseen by the positive correlation of interstitial infiltrates with *Coll IV* gene expression.

In control kidneys, both in the glomerular and tubulointerstitial compartments, *RAS* genes were only faintly detectable (Fig. 4), so that it was not possible to perform any regression analysis. What we can say is that this pattern of RAS activity is quite different from the one observed in diseased kidneys, namely in IgAN, where it appears as an activated system. That RAS is activated in IgAN (i.e., that Ang II peptide levels in the renal tissue are increased) was suggested by previous investigations [46, 47]. So far, only few studies examined the expression of *RAS* gene components in diseased human kidney and namely in IgAN. Wagner et al [14] surprisingly observed low renal renin mRNA levels in seven non-ACE inhibitor–treated patients with glomerulonephritis (three of whom had IgAN). In that study, the assay was performed

on whole homogenized renal cortex. However, in a different study by in situ hybridization, in five IgAN patients, the mesangium and tubules, as in other glomerular diseases but differently from the normal kidney, were found to express renin mRNA [45], a finding that is confirmed by our observation. That particular study, which used a semiquantitative grading of mRNAs detected by in situ hybridization, disclosed that *ACE* and *Agtg* were also overexpressed in both glomeruli and tubules in respect to normal kidneys [45], again confirming our data.

More recently, in isolated glomeruli from 22 glomerulonephritic subjects (11 were IgAN patients), Wagner et al [44], reported a reduced expression of the *AT1 receptor* gene, corroborating data on whole homogenated cortex in 37 glomerulonephritic patients (19 of which were IgAN). Although not significantly, an interesting trend toward higher *AT1 receptor* mRNA levels was observed in the Wagner et al study [44] in patients on ACE inhibitors, supporting data obtained in experimental models [48, 49]. The intrarenal down-regulation of the *AT1 receptor* gene possibly mirrors high intrarenal Ang II activity. The data by Lai et al [45], demonstrating in IgAN patients, overexpression of glomerular *renin* and *ACE* genes, and our findings also corroborate this interpretation.

In the normal nonfetal human kidney, *AT2 receptors* have been recognized by in situ hybridization in the adventitia of interlobular arteries in the renal cortex [50–52]. The localization of AT2 receptors in perivascular interstitial structures in the kidney suggests that these receptors may play a relevant physiologic role in the renal interstitium. In support of this notion, mice carrying a targeted disruption of the *AT2 receptor* gene develop a more extensive and severe interstitial fibrosis after a renal injury than mice with intact AT2 receptors [32]. However, our findings disclose the existence of AT2 receptors also in the glomerulus, a result not previously reported, thus suggesting that the functional balance between AT1 receptors and AT2 receptors is also working at adult human glomerular level at least in the diseased kidney of IgAN. Although the *AT2 receptor* gene was expressed at a low level since we observed it at a relatively high number of amplification cycles and four patients out of 20 were negative at the glomerular level (while all were positive in the tubulointerstitial compartment), it was only very scantly or not expressed at all in control kidneys (Fig. 4). This suggests that the *AT2 receptor* gene expression is a disease-dependent phenomenon as observed in the skin, myocardium, and the central and peripheral nervous system [53]. However, further studies (i.e., in situ hybridization studies) will be necessary to definitely confirm this finding.

Although it is easily comprehensible that the RAS is activated in nephropathies with established reduction of

the nephron mass, it might be difficult to recognize that it also occurs in inflammatory-immunologic nephropathies without significant reduction of renal function as in our patients. However, the fact that RAS is precociously activated in glomerulonephritis at the renal level has been clearly demonstrated in three different experimental models [13, 54, 55]. That RAS activation occurs early in glomerulonephritis and namely in IgAN is clinically remarkable since it might disclose the opportunity for early introduction of RAS inhibiting agents in the therapeutic armamentarium of non- or low-grade proteinuric, or nonhypertensive IgAN patients. The current use of these agents is generally restricted to IgAN hypertensive patients or to those with higher levels of proteinuria, or showing initial derangement in renal function.

The broad range of renal activation levels of RAS and namely of *ACE* (Fig. 2B) and *AT1 receptor* genes (not shown) in different people suggest that a variable susceptibility to ACE inhibitors or AT1 receptor blockers might exist and that as a consequence, the beneficial effect on progression may be largely variable, a guess which is confirmed by the variability in terms of reaching clinical end points in most clinical trials with these agents [56, 57]. The large variability in RAS expression at kidney level is not explained by this study. It is proposed that different polymorphisms of the *RAS* genes may have a weighty impact on the prognosis of IgAN. Such an influence should be associated, and most likely pathogenetically linked to the genetically controlled intrarenal expression levels of *RAS* genes [58]. According to this theory, RAS polymorphisms might be very important determinants of such variability.

CONCLUSION

This study demonstrates that a tight regulation of the intrarenal RAS exists in IgAN and that it follows the general rules disclosed in animal models. Moreover, RAS seems to be activated in the diseased kidney and it appears that such RAS activation drives a parallel stimulation of chemotaxis of inflammatory cells and of the TGF-β fibrogenic loop, particularly at the tubulointerstitial level, which have a significant impact on the renal pathology. These findings confirm the rationale for the early treatment with ACE inhibitors and AT1 receptor blockers in IgAN even in the absence of hypertension and/or severe proteinuria.

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