Renin Gene Expression in Human Kidney Biopsies From Patients With Glomerulonephritis or Graft Rejection

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The expression of renin mRNA was determined by a quantitative polymerase chain reaction assay in 27 human kidney samples: (1) 15 biopsies of patients with glomerulonephritis with or without angiotensin-converting enzyme inhibitor (ACEI) treatment; (2) biopsies of six renal allografts with graft rejection; and (3) six biopsy samples from unaffected parts of tumor nephrectomy specimens as controls. After isolation of mRNA, 0.5 to 1 μg of total RNA was used for reverse transcription to generate cDNA. The human renin gene was subsequently amplified by the use of two primers spanning the second and third exons. Renin expression was quantified with a renin cDNA mutant as the internal standard. It exhibited the same primer binding sites as the endogenous gene but carried a 155-basepair deletion, thus yielding a shorter amplification product. The number of glomeruli was counted by microscopic translumination immediately after biopsy (median, 9 per biopsy; range, 2 to 23). Renin mRNA was expressed as femtograms of renin mRNA per glomerulus. Renin gene expression was lower in glomerulonephritic patients without ACEI treatment compared with that in control tumor nephrectomy samples, i.e., 63 ± 20 (N = 7) versus 250 ± 50 fg (N = 6) of renin mRNA/glomerulus, (P < 0.02), although plasma renin concentration in the glomerulonephritic patients was in the normal range. Significantly higher renin mRNA expression was found in glomerulonephritic patients treated with ACEI, i.e., 210 ± 50 (N = 8) compared with 63 ± 20 (N = 7) fg of renin mRNA/glomerulus in patient not treated with ACEI (P < 0.02). Renin expression in allograft biopsies of cyclosporine-treated patients with chronic rejection was markedly higher than that in glomerulonephritis (630 ± 170 fg of renin mRNA/glomerulus (N = 6); P < 0.01).

The data document the feasibility of the quantitation of mRNA in small renal biopsy samples by the mutant template polymerase chain reaction technique. The finding of higher renin mRNA expression in patients on ACEI demonstrates the regulation of renin gene expression in the diseased human kidney.

Key Words: Renin-angiotensin system, renal disease, glomerulonephritis, angiotensin-converting enzyme inhibitor, polymerase chain reaction, transplantation

The renin-angiotensin system has been identified as a factor involved in the progression of chronic renal failure as well as in the development of hypertension of renal parenchymal disease. Studies on plasma renin activity in patients with renal disease documented that it was slightly elevated or at least inappropriately high in relation to blood pressure (BP) and exchangeable sodium (1,2). In models of renal damage, the renin-angiotensin system may contribute to intraglomerular hypertension. Angiotensin converting enzyme (ACE) inhibition has been shown to retard progressive renal failure (3).

This raises the issue of how renin gene expression is regulated in the renal disease of humans. To date, only data from animal models are available and indicate increased renin gene expression in isolated glomeruli of subtotal nephrectomized rats (4). After subtotal ligation of branches of the renal artery, scar tissue adjacent to necrotic areas exhibits an increase in renin mRNA message, whereas the mRNA levels are normal in remnant glomeruli that develop glomerulosclerosis (5).

In humans with ESRD, renin is readily detectable by immunohistochemical techniques (6). However, thus far, no information is available concerning the regulation of renin gene expression in diseased human kidneys. This is the result of technical reasons, because the size of tissue samples available for renal biopsies is too small to allow analysis of mRNA expression by conventional techniques. With the recent availability of the highly sensitive polymerase chain reaction (PCR), measurements of gene expression from very small tissue samples have become possible (7,8) and have been used in animal studies (see Reference 21). To quantitatively determine the amount of renal renin gene expression per glomerulus, we applied a modification of the PCR assay, coamplifying a deletion mutant of the human renin gene as internal standard (7). The expression of the renin gene could thus be measured in biopsy samples from patients with glomerulonephritis (GN) or transplant rejection.
Patients

Controls. Kidney samples were obtained from six tumor nephrectomy specimens (five women, one man; median age, 64 yr; range, 56 to 72 yr; systolic BP, 154 ± 4 mm Hg; diastolic BP, 94 ± 7 mm Hg; no treatment with ACE inhibitors [ACEI] or diuretics). Renal biopsies were taken at the time of surgery from sites remote from the tumor-bearing tissue (see Renal Biopsies section). Patients did not undergo thrombembolization of the renal artery.

GN. Fifteen patients with GN were examined (median age, 23 to 62 yr; 12 men; women); the patients had no dietary restriction. None of the glomerulonephritic patients was on steroids, immunosuppressive drugs, or contraceptives. Median 24-h urinary Na excretion before admission was 140 mmol/day (range, 68 to 300). Two groups were compared, i.e., patients with or without ACEI. Seven patients were not treated with ACEI; six of these were without antihypertensive treatment, and one used a cardioselective β-blocker. Diagnoses were immunoglobulin A nephritis (N = 3), Schoenlein-Henoch purpura (N = 1), epimembranous GN (N = 1) and nondescript mesangial scarring (N = 2). Eight patients had ACEI. Diagnoses were immunoglobulin A nephritis (N = 3), minimal change GN (N = 2), postinfectious GN (N = 1), and mesangial proliferative GN (N = 2).

Graft rejection. Biopsies were collected from six patients with kidney allografts, i.e., four men and two women (median age, 39 yr; range, 22 to 51), the patients were neither treated by diuretics or ACEI. For immunosuppression, all patients were placed on cyclosporine and prednisone. Only four patients required nifedpine as antihypertensive treatment. Systolic BP was 139 ± 5 mm Hg; diastolic BP was 89 ± 4 mm Hg. The biopsy was performed 2 months to 4 yr after transplantation. The histologic diagnoses were chronic interstitial rejection (N = 4) and acute interstitial rejection (N = 2).

Renal Biopsy

Three days before biopsy, BP was measured at 3-h intervals on the ward and patients were placed on a 100 mmol/day sodium-containing diet. One biopsy was taken for diagnostic purposes and one for measurements of renin gene expression after written informed consent was obtained from the patients before the biopsy. The procedure was approved by the local ethics committee. Biopsies were obtained from the left lower pole under ultrasound guidance (Toshiba Sonolayer, Toshiba Medical Systems, Neuss, Germany) with a Biopsy (Radiplast AB, Uppsala, Sweden) system with an 18-gauge (1.2-mm) needle. At the time of surgery, renal biopsies were obtained immediately after ligation of the renal artery with less than 10 min of warm ischemia time by a nephrologist scrubbed and in sterile gaunts present at the operating table. Biopsies were performed under direct visualization. The Biopsy needle was directed perpendicularly to the surface so that sampling conditions were similar to ultrasonically guided biopsies in renal patients.

The biopsy samples were placed on a sterile glass plate, and the glomcrull were immediately counted by the use of a Leitz SM-Lux (Leitz, Wetzlar Germany) microscope. Counting was performed by the same person in all cases. The procedure lasted less than 60 s. Subsequently, the specimens were placed in presterilized Eppendorf tubes and immersed in liquid nitrogen. Biopsy samples were stored at −80°C until further analysis.

Quantification of Renin Gene Expression

RNA isolation. Total RNA was isolated by a modification of the lithium chloride method (10). Total RNA isolated per biopsy was 0.7 to 19 μg, depending on the size of the sample. To avoid RNA degradation in biopsy samples, the biopsy cylinder was immediately placed in Eppendorf tubes after transillumination, frozen immediately thereafter in liquid nitrogen, and stored at −80°C. For RNA isolation, biopsy samples were kept on dry ice and then immersed in ice-cold lithium chloride. This prevented thawing of the samples before homogenization. In selected biopsies, degradation of total RNA was checked by gel electrophoresis after denaturation with 6 M glyoxal, 0.25 M dimethylsulfoxide, and 0.1 M NaH2PO4 (pH 7.0) at 50°C for 60 min.

Competitive PCR for Quantification of Human Tissue Renin mRNA. Renin mRNA was quantified in the presence of a defined concentration of a deletion mutant of the human renin gene as an internal standard, as described in Paul et al. (7). In brief, the deletion mutant of the human renin gene was obtained by the excision of a 155-base-pair (bp) fragment by Apol (restriction sites 1023 and 1178) and subsequent religation. The renin upstream and downstream primers used for PCR bind at 851 to 872 bp and 1206 to 1227 bp of the human renin gene, resulting in an amplification product for the endogenous renin gene of 376 bp and 221 bp for the mutant, respectively, with the same primer binding sites. The PCR primers were selected to span exon boundaries. A contamination of RNA samples with genomic DNA could therefore easily be detected by amplification of a larger PCR product than that expected for RNA. However, under the PCR conditions used, no DNA contamination band could be observed. Because, for PCR quantification a cDNA mutant and not an RNA mutant has been used, according to Siebert et al. (see Reference 19), we have previously determined the efficiency of reverse transcription by determining the recovery rate of incorporated radioactively labeled nucleotides added to the reverse transcription reaction mixture by trichloroacetic acid precipitation (7). Accounting for the efficiency of the reverse transcription, a factor of 0.51 was integrated in the calculation of renin mRNA per glomerulus (7): 0.5 to 1 μg of total RNA was reverse transcribed in the presence of 100 pmol of random hexamers for 45 min at 42°C. For quantification, 0.1 μg of human renin deletion mutant cDNA (referring to 5 × 106 molecules) were added to the reverse transcription sample and the mixture was serially diluted 1.25 times to establish a standard curve for the estimation of endogenous renin; a PCR was then performed on each sam-
ple. The PCR mixture contained 50 mM KCl; 20 mM Tris-HCl (pH 8.4); 2.5 mM MgCl₂, 10 μg/μL nuclease-free bovine serum albumin (Boehringer, Mannheim, Germany); 1 mM dATP, dCTP, dGTP, and dTTP; 25 pmol of upstream and downstream primers; and 3 U of Taq polymerase (Gibco BRL). PCR cycles were 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min for 28 cycles. After PCR, 25 μL of the dilution series samples was used for gel electrophoresis (1% agarose) and Southern hybridization on a nylon membrane (Pall, GB) by vacuum blotting as described by Hirsch et al. (11). Renin cDNA was hybridized to a [32P]dCTP radioactively labeled human renin cDNA HindIII, EcoRI fragment of 750 bp. Membranes were exposed after being washed for 20 min on a Phosphoirnager system (Fuji, Japan), and the amount of amplification products was analyzed as PSL/mm².

The autoradiographic signals were counted on a phosphoimager and plotted against the amount of total RNA as well as the number of mutant renin molecules present in the samples of the dilution series. A regression analysis was performed for the mutant and for the endogenous renin gene, and then the number of mutant molecules which corresponded to the same signal strength as the endogenous renin gene at a certain amount of total RNA was calculated. From that, the total femtograms of renin mRNA per sample was derived. Quantitative PCR determinations that resulted in a correlation coefficient on regression analysis of less than 0.8 were omitted.

Statistics

Results are expressed as means ± SE. Data were evaluated by the use of the t test. Statistical significance was accepted at P < 0.05.

RESULTS

Glomerular Counts

By the use of the microscopic transillumination technique and by focusing through the entire biopsy cylinder, glomeruli could be identified as spherical structures, the recognition of which was facilitated by the presence of erythrocytes within the tuft and in some cases within Bowman's capsule. The number of glomeruli per biopsy sample ranged from 2 to 23, with a median of 9 glomeruli. Glomerular counting by microscopic transillumination has been validated by comparing the results of a periodic acid-Schiff-stained biopsy specimen with the results of the preceding transillumination counting from tumor nephrectomy samples. Identical counts were found in 73% of the samples, and the maximum difference was one glomerulus or an average of 0.36 glomeruli.

Technique of Quantitative PCR Analysis of Renin mRNA in Human Renal Biopsies

For the measurement of renin gene expression, a deletion mutant of the endogenous renin gene was constructed (7) and included the same primer binding sites as the endogenous renin gene. Figure 1C shows the dilution series of the PCR after Southern hybridization. The lower band gives the mutant; the higher band represents the endogenous gene. It has previously been shown (12,13), that mutant and endogenous renin cDNA compete for the primers so that raising amounts of either one increasingly inhibits amplification of the counterpart. The amount of mutant cDNA that allows amplification of both the endogenous and the mutant cDNA had to be established. In reverse-transcribed cDNA from human kidney biopsies, 0.1 pg of renin mutant cDNA led to coamplification of both PCR products. At this mutant cDNA concentration, the Figure 1B insert gives an example of high endogenous renin expression with a stronger signal intensity for the endogenous gene, whereas in Figure 1C, the renin mutant cDNA signal is dominant. Because the amount of total cDNA as well as of mutant renin cDNA is known for each sample of the dilution series, the signal intensities of the internal standard can be used to calculate the amount of endogenous renin per total cDNA by the use of regression analysis. Figure 1D shows that, in renal biopsies, quantitative PCR is in the linear range of the test system. Evaluation of the stability of mRNA in biopsy samples of nephrectomy specimens showed unchanged renin mRNA levels at 1 and 3 min and a 29.7% loss at 5 min at room temperature. The efficiency of renin mRNA extraction was highly reproducible, as assessed by measurements of total RNA extracted per milligram of protein in 12 parallel samples from the same kidney (coefficient of variation, 18%). The correlation of renin mRNA per sample to the number of glomeruli was markedly better (r = 0.91; Figure 2A) than per microgram of total RNA (r = 0.67; Figure 2B) or other reference parameters, e.g., biopsy volume (r = 0.47), when control tumor nephrectomy samples were examined.

Renin mRNA in Biopsy Samples

Table 1 shows renin mRNA in biopsy samples from controls, patients with GN (with and without ACEI), and patients with allograft rejection. Renin mRNA was significantly lower in patients with GN than in controls. Patients with ACEI had significantly higher renin mRNA than patients without ACEI treatment. Patients with acute or chronic allograft rejection had significantly higher renin mRNA values than did GN patients. The values tended (P < 0.07) to be higher than in controls. No significant correlations were found to BP, renal function, and proteinuria (data not shown). Also, no obvious differences of renin mRNA in the various types of renal disease were noted, but admittedly, the numbers of patients are small.

The active PRC was determined with a monoclonal antibody against the active site of human renin. Seven patients with GN without ACEI treatment were examined after overnight supine rest before and after 25 mg of captopril po equilibrated on a 100 mM NaCl diet. Blood samples were drawn 60 (data not given) and 120 min after captopril. Preccaptopril PRC was 22.2 ± 6.4, and postcaptopril PRC was 33.8 ± 7.0 pg/mL. A comparison between renal renin gene expression and PRC did not show a correlation between renin mRNA
Figure 1. Quantitative PCR for renin in renal biopsies. After reverse transcription, renin mRNA is coamplified in the presence of a defined number of renin mutant cDNA. Before PCR, (a) 0.5- to 1-μg sample RNA and renin mutant cDNA mixture are serially diluted 1:2 resulting in decreasing signal intensities. (A) Southern hybridization of a dilution series. After determination of densitometric units (PSU/mm²), regression analysis of the amplification products is performed. Depending on the amount of renin mRNA per microgram of total RNA, the signal intensity of a certain number of mutant molecules corresponds to the amount of total RNA required for the endogenous renin mRNA to yield the same signal intensity. (B) Example for high renin mRNA concentration. At a given number of renin mutant cDNA molecules, the endogenous renin cDNA reaches the same signal intensity at a comparably low amount of total RNA. (C) Low renin concentration. For the same signal intensities, a higher amount of total RNA is required. (D) Linearity of the PCR amplification according to PCR cycle number. Toward the 40th cycle, the PCR assay reaches a plateau. However, at cycle 28, where the assay is performed, linearity is still present.

and the increment of active renin concentration before and after 25 mg of captopril in those patients with GN who had not been on ACEI. This is also true when maximal PRC is compared with renal renin gene expression per glomerulus (data not shown).

DISCUSSION

This study documents the feasibility of quantitating renin mRNA in human renal biopsies by use of the quantitative mutant template PCR technique. Renin expression in the kidney is highly restricted to the juxtaglomerular apparatus. We chose to count glomeruli because no housekeeping genes exist with expression (1) confined to the glomeruli and (2) not potentially altered by renal disease. Although this may not be strictly correct for all types of renal disease, it is commonly assumed that glomeruli are homogenously affected by glomerular disease and that a small biopsy sample is representative of glomerulonephritic lesions in the kidney (14). Undoubtedly, however, the extent to which individual glomeruli are affected differs. The assumption that renin expression is restricted to the juxtaglomerular apparatus may also not be true in all types of renal disease, e.g., in autosomal dominant polycystic kidney disease, renin synthesis has been demonstrated in tubular cells (15) and extraglomeru-
The PCR has been extremely useful for the detection and quantitation of the gene expression of nucleic acids in low abundance or small biologic samples, e.g., quantitation of viremia (17) or viral genome content in nonrenal biopsies (18). This can be extended to the kidney, where viral DNA can be detected in renal biopsies (19) or the expression of RNA indicating sclerosis (20,21) can be quantified.

To measure the amount of renin mRNA, a number of PCR assays have been developed (22,23). We used a deletion mutant of the human renin gene as an internal standard. Because of the sensitivity of the PCR, small alterations in the reaction conditions at the beginning of PCR may have a large effect on amplification efficiency (12). An internal standard avoids intersample variations of the PCR, because it is coamplified with the endogenous gene. Furthermore, the use of the same primer binding sites as the native human renin gene ensures identical amplification efficiency because of the same primer binding affinities (7), in contrast to approaches where reporter genes different from the native gene have been used (24). A deletion mutant cDNA of the human renin gene has been applied, because in prior experiments, the reproducibility of the reverse transcription reaction with random hexamers with an efficiency of 0.5 could be demonstrated. The reverse transcription reaction is self-terminated after the synthesis of cDNA along the mRNA template. After less than 30 min, no further increase in cDNA is noted and in the presence of 1 μg of RNA, 200 U of murine leukemia virus reverse transcriptase give a maximum of cDNA synthesis (7). These findings are in agreement with recent technical reports on the use of cDNA mutants for reverse tran-

**Table 1. Renin mRNA in kidney biopsy samples**

<table>
<thead>
<tr>
<th>Group</th>
<th>Renin mRNA (pg/glomerulus)</th>
<th>Serum Creatinine (mg/dL)</th>
<th>Proteinuria (g/day)</th>
<th>Systolic Blood Pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (N = 6)</td>
<td>250 ± 50</td>
<td>1.02 ± 0.1</td>
<td>Not done</td>
<td>154 ± 4</td>
</tr>
<tr>
<td>GN (N = 15)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Without ACEI (N = 7)</td>
<td>63 ± 20c</td>
<td>1.16 ± 0.14</td>
<td>1.77 ± 0.7</td>
<td>128 ± 2.7</td>
</tr>
<tr>
<td>With ACEI (N = 9)</td>
<td>210 ± 50c</td>
<td>1.62 ± 0.27</td>
<td>4.0 ± 0.9</td>
<td>143 ± 8.4</td>
</tr>
<tr>
<td>Allograft Rejection</td>
<td>630 ± 170c</td>
<td>1.94 ± 0.4</td>
<td>Not done</td>
<td>139 ± 5</td>
</tr>
</tbody>
</table>

*a* Significant difference from control, P<0.02.

*b* Significant difference from GN without ACEI, P<0.05.

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**Figure 2. Relationship between the counted number of glomeruli per biopsy sample and renal renin mRNA expression (A) and micrograms of total RNA (B) in control tumor nephrectomy samples. Glomerular count by microscopic transillumination and picograms of renin mRNA are more closely correlated in Panel A (r = 0.91) than in Panel B (r = 0.67).**

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lar arterioles (16). To account for the spatial distribution of renin mRNA in human kidney, we normalized renin mRNA to the number of glomeruli in the biopsy specimen. The variance was less for renin mRNA per glomerulus than for renin mRNA per total RNA, illustrating that it is advantageous to refer renin mRNA to the number of glomeruli as counted by transillumination microscopy.

The PCR has been extremely useful for the detection and quantitation of the gene expression of nucleic acids in low abundance or small biologic samples, e.g., quantitation of viremia (17) or viral genome content in nonrenal biopsies (18). This can be extended to the kidney, where viral DNA can be detected in renal biopsies (19) or the expression of RNA indicating sclerosis (20,21) can be quantified.
scriptase PCR for quantitative measurements (25). The linearity of the PCR beyond the 32nd cycle has been determined, indicating that the amplification of cDNA from human renal biopsies is in the exponential phase under the experimental conditions. To avoid artefacts from low-amplification efficiencies in individual PCR samples, from DNA hybridization, or from phosphorimager measurement, a dilution series of every single reverse-transcribed mRNA was performed before PCR. By these means, the final determination of renin mRNA concentration could be calculated from a regression analysis instead from a single point of measurement. Reverse transcription PCR was repeated when the correlation coefficient was less than 0.8. By this approach, the coefficient of variation of this method could be kept below 15% (7). Repeated measurements from single biopsies in a time frame between 4 wk and 1 yr also indicate that mRNA from renal biopsies is stable for prolonged periods of time if the samples are stored at −80°C as recommended in Reference 26.

Despite the traumatization of renal tissue during biopsy, a comparison of cylinders taken and left for 0, 60, 120, and 180 s at room temperature showed that the amplified renin mRNA signal remains constant for approximately 3 min. This time frame, i.e., 60 s, is well below the time required for the counting of the glomeruli.

We emphasize the contrasting behavior of circulating renin and tissue-level renin expression. No relationship was noted between baseline PRC (data not given) and the increment of postcaptopril PRC on the one hand and renin mRNA on the other hand. This illustrates that the tissue-level expression of renin mRNA cannot reliably be predicted from measurements of circulating components of the renin system. The reasons for this discrepancy were not investigated but may include problems of altered prorenin to renin conversion and the efficacy of translation and posttranslational processing.

The findings of low renin mRNA in patients with GN without ACEi treatment does not necessarily imply that the tissue level of renin is appropriate. The activity of the renin system can only be interpreted in the context of BP and body sodium. Increased body sodium per standard body surface was found in normotensive and mildly hypertensive individuals with GN (1). In the limited number of patients in our study, no relationship was found between BP and renin mRNA.

The above results of low renin mRNA per glomerulus in GN contrast with previous reports on high renin message per glomerulus in rats with renal ablation (14). The model used, i.e., vascular ligation, is known to cause renal ischemia. PRC is considerably higher after renal ablation by vascular ligation than by surgical resection (27). The notion of an ischemia-related increase in renin mRNA is further supported by the observation of increased renin gene expression in adjacent scar tissue. The discrepancy illustrates that renin gene expression may vary between different renal diseases so that findings in one disease state can not necessarily be extrapolated to others.

Patients on ACEi had consistently higher renin mRNA than patients without ACEi; values in the two groups did not overlap. This observation would be consistent with preserved regulation of renin mRNA even in the diseased kidney or at least directionally appropriate changes, but it cannot completely be excluded that patients requiring ACEi had more severe GN and thereby more marked activation of the renin system. It remains unsolved whether the increase in renal renin gene expression is appropriate for the given dosage of ACE inhibition. As a preliminary approach, we also explored histologic parameters, including vascular lesions, using a score system. No relationship between any of the histologic parameters and renin mRNA was noted (data not given), but clearly, more extended studies in larger populations with more homogeneous renal pathology are required to resolve this important issue.

Another interesting aspect of the study is the observation that renin mRNA was higher in biopsies of allografts with rejection. In the allograft with rejection, renin mRNA may be stimulated via an action of cytokines on the juxtaglomerular apparatus or via ischemia from vasculitic lesions. However, all transplanted patients were treated with cyclosporin A and/or steroids. Because cyclosporin A is known to raise renin gene expression (28,29), it may be responsible for the higher renal renin mRNA levels. Effects of steroids on renin mRNA are more controversial (30,31). Rapid changes in the PRC have been reported in animals subjected to anesthesia (32). Similarly, anesthesia for patients undergoing tumor nephrectomy may influence the PRC; therefore, subtle effects on renal renin mRNA expression are therefore not excluded, but changes on the transcriptional level are definitively unlikely, given the brief duration of anesthesia.

Obviously, the effect of the activation of the renin system on the kidney cannot be accurately assessed by the measurement of renin mRNA alone. The local availability and functional consequences of angiotensin II may depend, among others, on ACE concentration or angiotensin II receptor density. It would be naive to conclude from the finding of low renin mRNA that renin is not important in, or related to, the glomerulonephritic lesion.

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