Age-Related Increase in Expression of TGF-β1 in the Rat Kidney: Relationship to Morphologic Changes

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Abstract. In the kidney, aging is characterized by the development of structural changes, including glomerulosclerosis and interstitial fibrosis. Transforming growth factor-β1 (TGF-β1) is known to play a critical role in the genesis of these alterations in pathologic conditions. The present experiments were designed to test the hypothesis that TGF-β1 may be involved in the development of age-related histopathologic changes in rat kidney, and that captopril, an angiotensin-converting enzyme inhibitor, may influence the progression of glomerular and interstitial lesions. In this study, 3-, 18-, 24-, and 30-mo-old rats were examined, and an age-related increase in urinary protein excretion was found; plasma creatinine and systolic BP did not change. Significant structural changes, including glomerular sclerosis and interstitial fibrosis, were found in the group of aged rats (24- and 30-mo-old). Immunostaining for TGF-β in the renal cortex interstitium was increased in the group of 24-mo-old rats, with a parallel increase in TGF-β1 mRNA expression, measured with reverse-transcription PCR. Captopril-treated animals showed a statistically significant decrease in urinary protein excretion but no significant changes in BP. Moreover, captopril reduced the extent of interstitial fibrosis, but did not affect the degree of glomerulosclerosis. A significant inhibition of TGF-β1 mRNA expression was observed in the captopril-treated animals. These findings suggest that TGF-β1 may act as a fibrogenic growth factor that could be responsible, at least partially, for the renal interstitial fibrosis associated with aging. Treatment with captopril might delay the progression of these lesions. (J Am Soc Nephrol 9: 782–791, 1998)

In recent years, particular attention has been paid to the analysis of the mechanisms involved in the development of progressive kidney disease. Most studies in this field have dealt with the pathogenesis of glomerulosclerosis, one of the most characteristic lesions (1–3), but the importance of interstitial fibrosis is being increasingly recognized (4). Regardless of the nature of kidney disease, both glomerulosclerosis and interstitial fibrosis are considered common final pathways in the development of progressive renal damage (1–4).

Among the mechanisms underlying renal scarring, transforming growth factor-β1 (TGF-β1) is known to play a critical role in the regulation of this process (2,5,6). At the glomerular level, the importance of TGF-β1 in the genesis of glomerulosclerosis has been clarified in recent years (2). Since the initial description by Border *et al.* (7) of the importance of this growth factor in the development of glomerulosclerosis in an experimental model of immune glomerular injury, different reports have documented the relevance of TGF-β1 in other models of progressive glomerular sclerosis (2,8–11). In addition, this cytokine also seems to play a significant role in the induction and maintenance of interstitial fibrosis (8,12). The role of TGF-β1 in nephrosclerosis is readily understood in view of the well defined role of this peptide in the regulation of cell proliferation (6,13), as well as in matrix synthesis and degradation (2,6,14–17). TGF-β1 stimulates gene transcription and the production of collagen I, III, V, and VI, as well as the production of fibronectin, tenascin, osteonectin, osteopontin, thrombospondin, and matrix glycosaminoglycans (15). It has also been reported to inhibit collagenase and stromelysin transcription (16) and to stimulate the synthesis of metalloproteinase inhibitors (17). Although it is generally accepted that sustained TGF-β1 expression underlies the development of progressive renal fibrosis, the factors that cause TGF-β1 overexpression are not completely understood.

Aging is considered a physiologic process, but it is characterized in the kidney by the development of structural changes, including different forms of tissue scarring (18–20) that resemble those observed in pathologic situations (21). Therefore, in the present study, we tested the hypothesis that TGF-β1 may be involved in age-related renal progressive interstitial fibrosis and glomerulosclerosis in the rat. Our results show increased expression of TGF-β1 in the kidney of aging rats, and thereby

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provide further evidence for a causative role of TGF-β1 in age-related morphologic changes in rats.

Materials and Methods

Experimental Design

Male Wistar rats were housed in a temperature-controlled room (22°C ± 2) on a 14/10 h light/dark cycle under specific pathogen-free conditions and with free access to food and water. Rats of different ages (3-, 18-, 24-, and 30-mo-old, $n = 10$ in each group) were used for the experiments. In addition, ten 18-mo-old rats were treated with captopril (100 mg/L in the drinking water, approximately 10 mg/kg per d) (22) for 6 mo and then studied at the end of this period. In these captopril-treated rats, BP measurements and urine samples were also obtained, as described below, before starting the treatment.

Seven days before the end of the experimental period, BP was measured by the tail-cuff method in conscious animals. Two days later, the rats were placed in individual metabolic cages, and two 24-h urine samples were collected (days 4 and 5). Daily urine production was measured, and samples were centrifuged (10 min, 1500 × *g*) to remove contaminants and then stored at −20°C until analysis for protein content (23). The animals were anesthetized with ether, and blood was taken from the lower aorta into tubes containing 7.5% ethylenediamine tetra-acetic acid. Samples were centrifuged (20 min, 1500 × *g*), and aliquots of plasma were stored at −20°C until creatinine analysis. One kidney was removed and processed for histologic examination. A piece of the renal cortex from the other kidney was collected in a sterile propylene tube containing a denaturing solution for total mRNA extraction.

Renal Histopathology and Immunohistochemistry

For histologic examinations, we prepared buffered 4% formaldehyde-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin, Masson's trichrome, or periodic acid-Schiff. In addition, digital image analysis was done in paraffin-embedded tissue stained with Sirius red. In each sample, we analyzed 20 microscope fields each for interstitial and glomerular area. The images were captured using a black-and-white charge-coupled device video camera (Vidamax CCD BCD-700) coupled to an Olympus Optical BH-2 microscope (Tokyo, Japan) and digitized at 8 bits intensity resolution (256 grey levels) and with a global magnification of ×200. Optical image size was of 44221.34 mm^5 for a 256-×-256 square pixel image, resulting in an optical resolution of 0.67 mm^5/pixel.

Image processing and analysis were performed with MS-DOS 6.2 (Microsoft Corp., Redmond, WA) in an MS-Windows 3.11 environment (Microsoft) through a specific automatic application, Fibrosis HR (Master Diagnostica, Granada, Spain). This method permits accurate quantification of mesangial matrix, glomerular morphology, and interstitial fibrosis in a minimum of time (24). Interstitial fibrosis was quantified as percentage of interstitial fibrosis area with respect to the whole interstitial area measured. If mesangial matrix expansion was present, an index was calculated as a function of total glomerular area (Mesangial matrix expansion index = Mesangial area/Glomerular area). Glomerular sclerosis was quantified as percentage number of completely sclerosed glomeruli with respect to the total number of evaluated glomeruli.

Immunostaining was done on paraffin-embedded sections, using the streptavidin-biotin-phosphatase alkaline technique and a primary monoclonal antibody directed against an epitope of TGF-β1 and -β2 (clone 1D11.16, Genzyme Diagnostic, Cambridge, MA) (25), which recognizes these molecules in human, rat, and mouse tissues. Activity of the 1D11.16 antibody was confirmed by the neutralization of TGF-β-induced antiproliferative activity in an MV1Lu mink lung epithelial cell bioassay (26), and the specificity of this antibody in detecting TGF-β protein with the streptavidin-biotin-phosphatase alkaline technique was demonstrated previously in immunohistochemical assays (27). Briefly, 4-μm paraffin-embedded sections were cleared and rehydrated. After two 3-min washes in Tris-buffered saline (TBS), nonspecific reactivity was blocked with normal rabbit serum at 20%. The sections were incubated with the primary antibody, diluted at 1:50 in 0.1% bovine serum albumin (BSA), in a moist chamber at 41°C for 16 h. Subsequently, sections were washed three times in TBS and incubated for 30 min at room temperature with biotinylated anti-mouse goat immunoglobulin (Dakopatts, Glostrup, Denmark) at 1:50 dilution in TBS. After extensive washes, slides were incubated with the streptavidin-alkaline phosphatase complex at 1:100 dilution (Dakopatts) for 30 min at room temperature, washed, and incubated in the chromogenic substrate fast red TR salt (Sigma, St. Louis, MO) under microscopic control until the color signal appeared. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma) at a concentration of 25 mg/ml before incubation with chromogenic substrate. Samples were then washed in water, counterstained with Mayer's hematoxylin, and mounted with Aquatex (Merck, Darmstadt, Germany). Negative controls were run with an IgG1 isotypic primary antibody diluted at 1:50 in 0.1% BSA (Master Diagnostica) and a section immunostaining with primary antibody absorbed with 0.5 ng/ml ultrapure nature human TGF-β1 (Genzyme Diagnostic) diluted in 0.1% BSA as described (25).

Immunostaining for TGF-β was evaluated by two experienced pathologists as follows: 1 = mild or normal, 2 = moderate, and 3 = intense. The observers were blind to the age group and treatment of specimens. Slides were scored independently at the tubular, periglomerular, and interstitial level, and the final score was the mean of the two evaluations. A total score was also defined as the sum of the scores of the three histologic areas. Intraglomerular staining was negative in every case. As a positive internal standard for immunostaining, we used the positivity of the adventitial connective tissue of the arcuate and small arteries.

Mild immunostaining in the tubules was defined as the absence of staining, or positivity in isolated tubules. Staining was considered moderate when three or fewer positive tubules were seen per ×400 microscopic field, and intense when the number of positive tubules per field was higher. At the periglomerular level, mild immunostaining was defined as the positivity seen in sections from 3-mo-old control animals (see Results). Staining was considered moderate when Bowman's capsule was clearly delimited and there was immunostaining in medullary rays with spread to the interstitial tissue between adjacent tubules. Intense staining was defined as widespread positivity involving the adventitial connective tissue of the afferent arteriole. In the interstitium, immunostaining was considered mild, moderate, or intense according to the intensity of positivity in the peritubular connective tissue.

Measurement of TGF-β1 mRNA Levels Using Reverse-Transcription PCR

TGF-β1 mRNA expression in the rat kidney cortex was measured by using reverse transcription (RT)-PCR, with two different techniques. A semiquantitative procedure was first performed by analyzing TGF-β1 and GAPDH mRNA expression in the same sample, considering the latter as a housekeeping gene (28). This was followed by quantitative competitive PCR assay (29). In every case, total RNA was extracted from rat kidney cortex according to the method of Chomczynski and Sacchi (30), and both the quality and the quantity

of the RNA were verified by ethidium bromide staining of rRNA bands on an agarose minigel.

The upstream and downstream TGF-β1 primers were 5′-CTT CAG CTC CAC AGA GAA GAA CTG C-3′ and 3′-CAC GAT CAT GTT GGA CAA CTG CTC C-5′, respectively, which yielded a single band corresponding to a 298-bp cDNA fragment (31). Analysis with cycle sequencing revealed that the sequence was identical to position 1266–1564 in rat TGF-β1 cDNA. The upstream and downstream GAPDH primer sequences were 5′-GTA AAG GGT CGG TGT CAA CGG ATT T-3′ and 5′-CAC AGT CTT CTG AGT GGC AGT GAT-3′, respectively, which yielded a single band corresponding to a 558-bp cDNA fragment (28). Analysis with cycle sequencing revealed that the sequence was identical to position 3–561 in rat GADPH cDNA.

One microgram of total RNA was reverse-transcribed by incubating at 42°C for 30 min (RNA PCR kit from Perkins-Elmer, Roche Molecular Systems Inc., Branchburg, NJ). In the semiquantitative experiments, the reaction product was amplified by PCR, using the TGF-β1 and GAPDH primers together. For quantitative PCR, the cDNA obtained after the RT reaction was amplified with the TGF-β1 primers in the presence of increasing amounts of a DNA construct that competes for the oligonucleotide primers' binding sites. The DNA competitor was synthesized using a PCR mimic construction kit (Clontech Laboratories, Palo Alto, CA) following the manufacturer's instructions. The DNA competitor was designed to produce a PCR amplification product of 598 bp, so that it could be easily identified from the 298-bp PCR product of TGF-β1 cDNA on an agarose gel.

In both semiquantitative and quantitative experiments, PCR (MJ Research Inc., Watertown, MA) was programmed for 35 cycles at 55°C. The resulting ethidium bromide-staining gel was imaged using an Image-store Colour OneScanner (Apple Computer), and analyzed using National Institutes of Health Image 1.55 Software. For the semiquantitative measurements, the TGF-β1/GAPDH product ratio was calculated and considered an index of TGF-β1 mRNA expression. In quantitative PCR, the ratio of TGF-β1 product to DNA competitor standard was plotted against the attomolar concentration of the DNA competitor (29). Data were expressed as attomolar TGF-β1 cDNA concentration per milligram of total RNA. Contamination was ruled out by the fact that PCR was negative when the reaction was performed without a prior RT reaction.

Statistical Analyses

Data were expressed as mean ± SEM. The normality of each distribution of values was assessed by the Kolmogorov–Smirnov test. Comparisons were performed by one-way ANOVA and the Newman–Keuls multiple comparison test, except for the immunostaining values. Because these data did not show a normal distribution, they were compared by the Kruskal–Wallis test. $P < 0.05$ was considered statistically significant.

Results

Table 1 shows that urinary protein excretion in Wistar rats increased progressively with aging. In contrast, no changes were detected in systolic BP or in plasma creatinine levels in rats of different ages. The appearance under light microscope of the renal cortex of 3- and 24-mo-old rats is shown in Figure 1. As expected, severe structural changes were found in the group of aged rats, all of which also had proteinuria. Increased mesangial matrix with glomerular sclerosis (Figure 1, C and D), cystic appearance of some glomeruli with tuft atrophy (Figure 1D), tubular atrophy with thyroidization and thickening of the basement membrane in both tubular and capsular structures (Figure 1, C and E), and interstitial fibrosis (Figure 1F) were observed. Hypercellularity was not detected. The quantitative analysis of the most prominent morphologic changes in the 24-mo-old rats with respect to the 3-mo-old group is summarized in Figure 2. The percentage of sclerosed glomeruli increased significantly with aging. In addition, the mesangial matrix expansion index and interstitial fibrosis, measured quantitatively as described in Materials and Methods, were also increased in the 24-mo-old rats. In both cases, the differences were statistically significant.

Immunostaining for TGF-β in the group of young rats (3 mo) was mild and restricted to interstitial areas and Bowman's capsule, without tubular cell staining (Figure 3B). In contrast, the group of 24-mo-old rats (Figure 3, C through E) showed a marked increase in immunostaining for TGF-β in the cytoplasm of tubular cells (Figure 3D) and the periglomerular and peritubular interstitium (Figure 3, C and E). In the latter, immunostaining was more evident in areas of interstitial fibrosis. The semiquantitative evaluation of immunostaining demonstrated that differences in tissue TGF-β content were significant only in the interstitium (Figure 4), whereas the total score of immunostaining was also highest in 24-mo-old rats (mean total score of 3-mo-old rats: 3.9 ± 0.4 *versus* mean total score of 24-mo-old rats: 5.2 ± 0.5, $P < 0.05$).

The analysis of TGF-β1 mRNA content by RT-PCR showed that TGF-β1 mRNA increased significantly with aging. The top panel of Figure 5 shows the ethidium bromide staining of

Table 1. Age-dependent changes in systolic BP, plasma creatinine concentration, and urine protein excretion in Wistar rats[a]

Variable	Age (mo)			
	3	18	24	30
Systolic BP (mmHg)	144 ± 5.0	146 ± 7.0	143 ± 9.0	151 ± 9.0
Plasma creatinine (mg/dl)	0.60 ± 0.02	0.63 ± 0.07	0.62 ± 0.04	0.66 ± 0.06
Urine protein excretion (mg/d)	23 ± 3.0	163 ± 30[b]	310 ± 45[c]	461 ± 48[d]

[a] Results are the mean ± SEM of 10 animals.
[b] $P < 0.05$ *versus* 3-mo-old rats.
[c] $P < 0.05$ *versus* 3- and 18-mo-old rats.
[d] $P < 0.05$ *versus* 3-, 18-, and 24-mo-old rats.

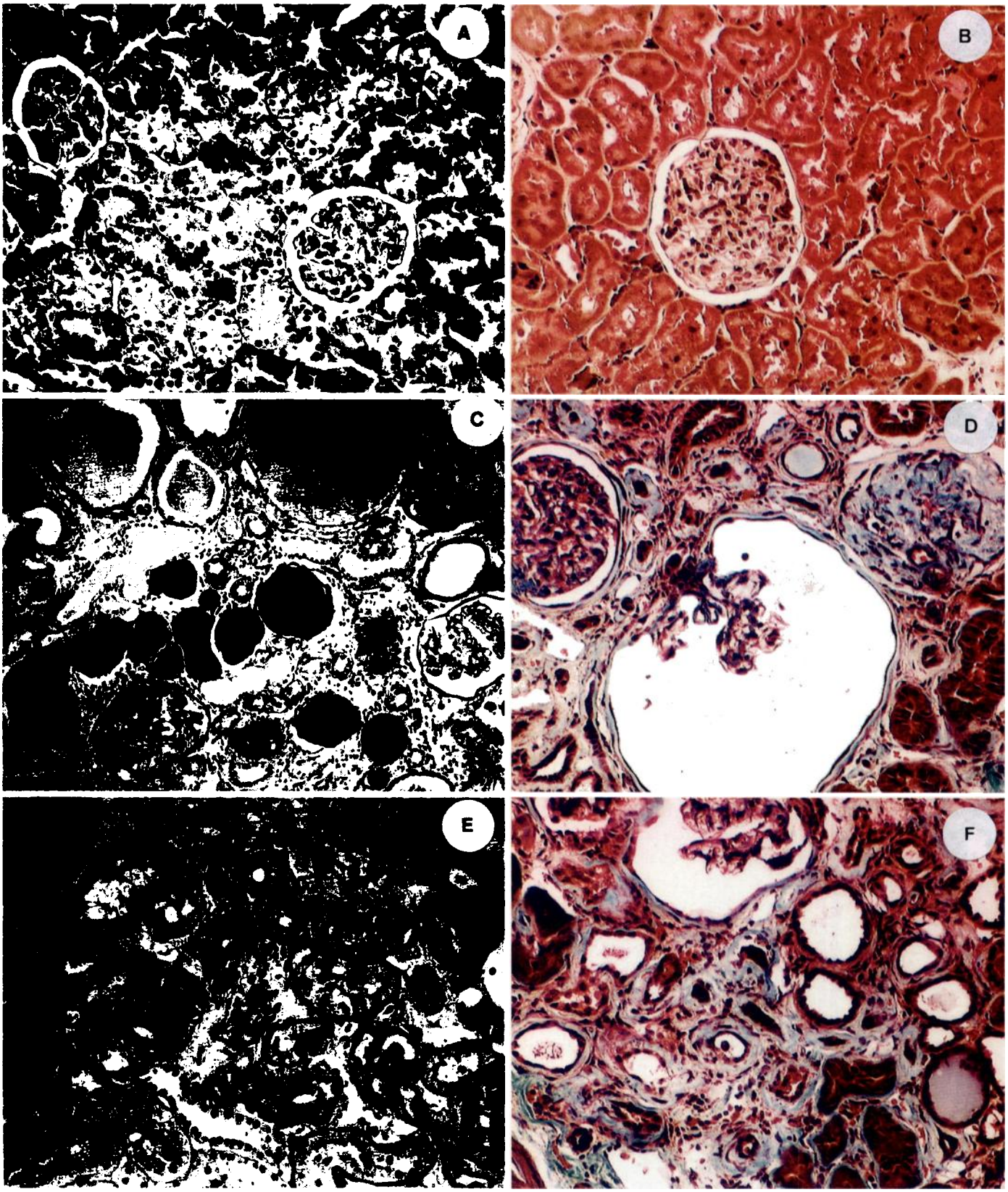


Figure 1. Histopathologic features in 3- and 24-mo-old rats. (A and B) Morphologic appearance of the renal cortex in 3-mo-old rats (hematoxylin & eosin and Masson's trichrome stain, ×200). Note the lack of interstitial and glomerular changes. (C through F) Histologic lesions of the renal cortex in 24-mo-old rats (Periodic acid-Schiff [C and E] and Masson's trichrome stain [D and F]). (C) Tubular atrophy with thyroidization (×100). (D) Glomeruli with cystic appearance and sclerosis (×200). (E) Collapse of some glomerular tufts and reduplication of basal membranes (×200). (F) Interstitial fibrosis (×200).

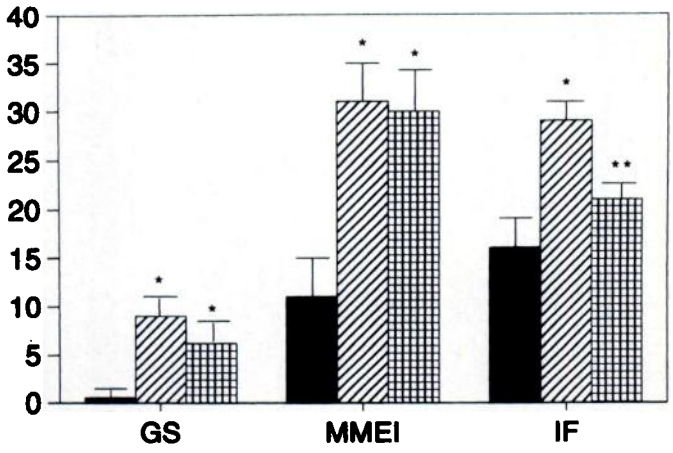


Figure 2. Quantitative analysis of the morphologic changes of 3-mo-old (■) and 24-mo-old (▨) rats, as well as of captopril-treated 24-mo-old rats (▦). Results are the mean ± SEM of 10 animals. GS, glomerular sclerosis, measured as percentage number of completely sclerosed glomeruli with respect to the total number of evaluated glomeruli; MMEI, mesangial matrix expansion index, measured as the percentage ratio between the mesangial area and its respective glomerular area (for more details, see Materials and Methods); IF, interstitial fibrosis, measured as percentage of fibrosis at the interstitium with respect to the complete interstitial area measured. *$P <$ 0.05 *versus* 3-mo-old rats. **$P < 0.05$ *versus* 3- and 24-mo-old rats.

a characteristic semiquantitative experiment. GAPDH and TGF-β1 were amplified together, and the amplification products for a particular sample were separated in the same lane. The ratio between the two densitometric signals was calculated for each rat, and the mean values of this parameter in the different groups of animals are summarized in the bottom panel of Figure 5. A significant increase in TGF-β1 mRNA levels appeared in 18-mo-old rats and remained thereafter (Figure 5). Because the standard RT-PCR technique is extremely efficient in detecting mRNA but cannot accurately quantify it, a specific DNA competitor, or "mimic," for TGF-β1 was developed for use in a competitive PCR procedure. Competitive RT-PCR clearly confirmed the age-related increase in TGF-β1 mRNA levels in 24-mo-old rats. The top panel of Figure 6 shows the characteristic pattern of response in competitive PCR in tissues from 3- and 24-mo-old rats. When data from the different animals were pooled, TGF-β1 mRNA levels in 24-mo-old rats were found to be five times higher than in young animals (Figure 6, bottom panel).

In 24-mo-old rats treated with captopril, urinary protein excretion was significantly lower than in nontreated animals of the same age (24-mo-old control rats: 466 ± 64 mg/d; 24-mo-old captopril-treated rats: 165 ± 23 mg/d, $P < 0.05$), whereas the urinary protein excretion before starting the treatment (172 ± 26 mg/d) was not different with respect to the animals of the same age that did not receive captopril (163 ± 30 mg/d). There was no difference between the two groups in systolic BP or plasma creatinine level (data not shown). The drug modified neither the percentage of glomerulosclerosis nor mesangial matrix expansion, but did significantly reduce interstitial fibrosis in 24-mo-old rats (Figure 2). In some 24-mo-old rats, captopril decreased the TGF-β immunostaining (Figure 3F), but no differences were detected in the intensity of TGF-β immunostaining between treated and nontreated 24-mo-old rats when all animals were considered (Figure 4). However, the results of the quantitative (Figure 7) RT-PCR procedures showed that captopril significantly reduced TGF-β1 mRNA expression in the kidney cortex of 24-mo-old rats compared with untreated animals of the same age.

Discussion

Glomerulosclerosis and tubulointerstitial fibrosis are hallmarks of progressive forms of renal disease of diverse etiologies, and are frequently present in kidneys from aged animals and humans. Age-associated renal changes include glomerular basement membrane thickening with tubular atrophy, interstitial fibrosis, and increases in the mesangial matrix of the glomeruli with glomerulosclerosis (18–21). A previous report by Abrass *et al.* (18) demonstrated that interstitial fibrosis precedes the development of sclerotic glomeruli, tubular atrophy, or the accumulation of interstitial collagen. Interestingly, those authors found a particular pattern of extracellular matrix deposition in the aged kidney and identified fibronectin and thrombospondin as components of the deposits. Although collagen I, III, and IV and laminin were present adjacent to areas of tubular atrophy, they did not contribute to interstitial fibrosis. These changes in extracellular matrix composition distinguish kidney aging from other sclerotic forms of renal disease (18).

The present results are compatible with previous descriptions of age-related kidney changes (18–21). We measured renal scarring with a quantitative image analysis method (24), which provides numeric data on the morphologic changes. This procedure made it possible to demonstrate not only that the number of sclerotic glomeruli increased with age, but also that the degree of mesangial matrix expansion was the greatest in 24-mo-old rats. The same quantitative procedure was used to measure interstitial fibrosis, which also increased in aged animals. The morphologic changes seem to be independent of BP, because systolic BP remained unchanged throughout the study in untreated animals. This result is compatible with the fact that Wistar rats are characteristically normotensive, even in old age (32). Baylis (32) demonstrated age-related glomerular changes in this breed that were not associated with hypertension. Thus, the results in our aged rats are unlikely to be dependent on changes in BP.

We also evaluated kidney TGF-β1 levels in young and aged animals. Many studies have documented TGF-β1 expression in the glomeruli and interstitia during the progressive sclerosis that characterizes many kidney diseases (8–11,33,34). However, few descriptions of the exact immunohistochemical location of this molecule in kidney tissues have been published (35). Studies with RT-PCR in nephrons isolated by microdissection have postulated that this factor is produced constantly by each element of the nephron (glomerulus and all parts of the tubules and arterioles) (36). However, a recent report based on immunohistochemical analyses similar to those used here

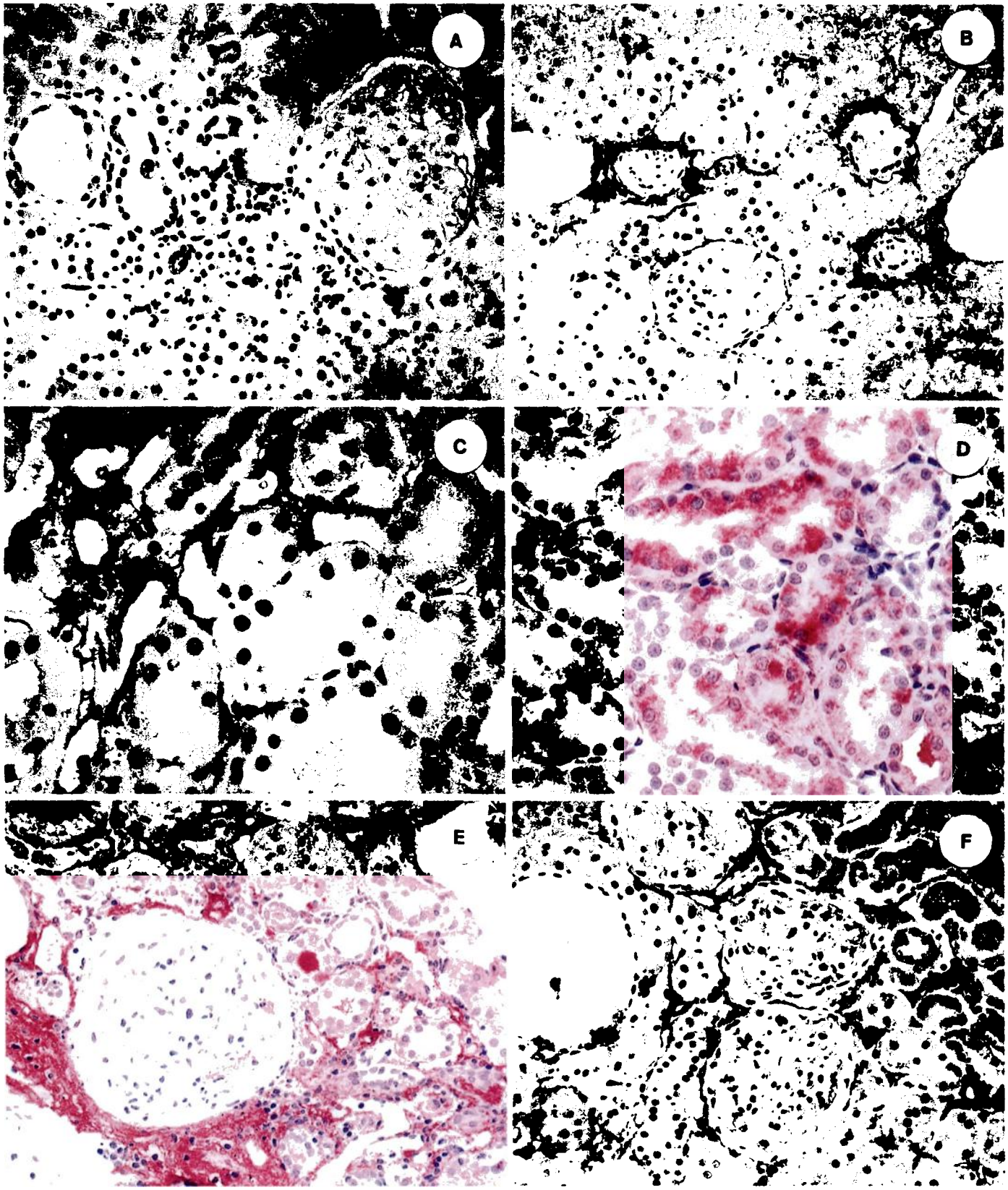


Figure 3. Transforming growth factor-β (TGF-β) immunostaining in 3- and 24-mo-old rats. (A and B) Negative control with primary antibody absorbed with ultrapure human TGF-β1 (A) and normal pattern (B) of immunostaining in 3-mo-old rats (streptavidin-biotin-alkaline phosphatase, ×200). Note the absence of normal immunostaining for TGF-β (A), and characteristic perivascular and periglomerular staining and the mild interstitial deposits of TGF-β (B). (C through E) Interstitial, tubular, and periglomerular immunostaining in 24-mo-old rats (streptavidin-biotin-alkaline phosphatase). Note in C (×400) the interstitial staining for TGF-β, in D (×200) the presence of more than three tubules with immunostaining for TGF-β (intense tubular pattern), and in E (×200) strong interstitial and periglomerular staining for TGF-β in areas with severe fibrosis. (F) TGF-β immunostaining in the renal cortex of a captopril-treated 24-mo-old rat (×200). Note the similar appearance to control rats.

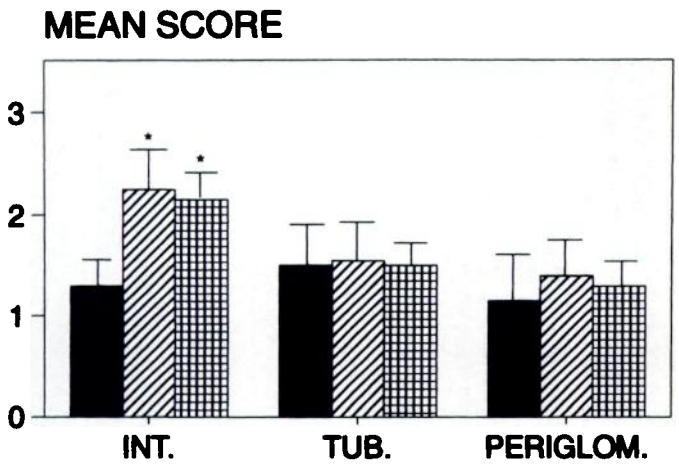


Figure 4. Semiquantitative analysis of TGF-β immunostaining in the renal cortex of 3-mo-old (■) and 24-mo-old (▨) rats, and in captopril-treated 24-mo-old rats (▦). Results are the mean ± SEM of 10 animals. INT., interstitial immunostaining; TUB., tubular immunostaining; PERIGLOM., periglomerular immunostaining. For more details on the quantification method, see Materials and Methods. $*P < 0.05$ *versus* 3-mo-old rats.

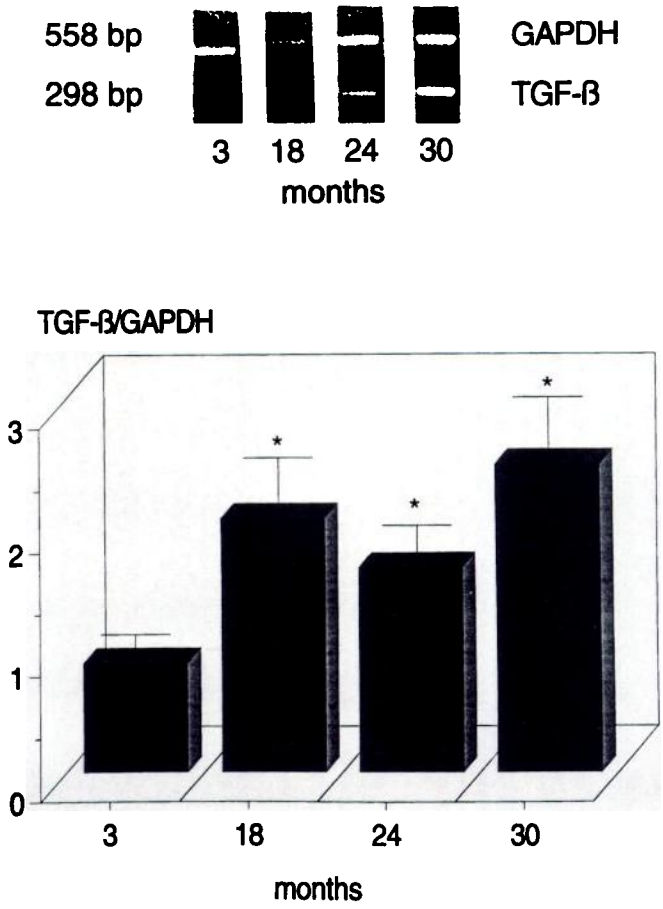


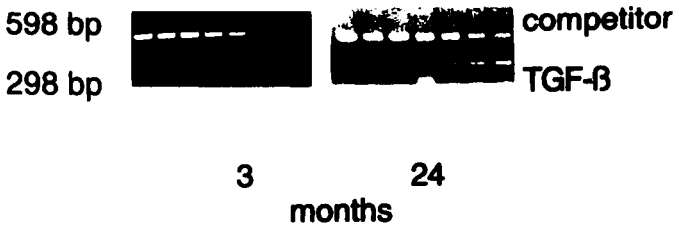
Figure 5. Semiquantitative measurement of TGF-β1 mRNA expression by reverse-transcription (RT)-PCR in rats of different ages. (Top) Characteristic agarose gel electrophoretic appearance of the simultaneous amplification products of GAPDH and TGF-β1 in one representative animal from each group. Size of amplified products is indicated in base pairs (bp). (Bottom) Semiquantitative analysis of the ratios between the densitometric signals of TGF-β1 and GAPDH in the four experimental groups. Results are the mean ± SEM of 10 animals. $*P < 0.05$ *versus* 3-mo-old rats.

failed to demonstrate a significant amount of TGF-β1 in the glomeruli, except in some juxtaglomerular apparatus. Instead, immunostaining was more intense in the periadventitial septal spaces around the interlobular arteries and arterioles, in peritubular interstitial spaces, straight vessels, renal medulla, periglomerular interstitial spaces, and in isolated tubules (37).

Our findings of an age-related increase of TGF-β1 mRNA levels associated with an increase of glomerular sclerosis and interstitial fibrosis and immunostaining for this cytokine in the kidney strongly suggest that TGF-β1 may act as a fibrogenic factor, which could be responsible, at least in part, for the progressive pathologic fibrotic changes in the kidney during aging. However, in the case of glomerulosclerosis, the tissue distribution of immunoreactive TGF-β1 does not support this hypothesis, because this structural alteration appeared even in the absence of glomerular TGF-β-positive immunostaining. In any case, we cannot completely rule out a relationship between glomerulosclerosis and TGF-β1 in aging, because the immunohistochemical methods used could have a lower sensitivity than that of the molecular techniques, and a lack of TGF-β detection may not be interpreted as being absent in glomeruli. In addition, TGF-β1 induction may occur early (between 24 h and 2 wk), as described in other studies using different experimental models (37,38), and may precede the development of fibrosis. Thus, in our model, this early event may have been masked by its effect on collagen formation.

At this point, a very important aspect of TGF-β must be considered. This growth factor is mostly present as its inactive form in tissues, and its activity depends not only on the amount of cytokine, but also on the degree of activation of the protein as well as the level of expression of its specific receptors (6). Moreover, TGF-β is inactivated by decorin (39), and it has been recently suggested that a deficiency in decorin could be responsible for the progression of interstitial fibrosis in chronic renal diseases (40). Therefore, it is not possible to directly relate the degree of TGF-β mRNA expression or the levels of immunoreactive TGF-β to the activity of the growth factor. Hence, the above-mentioned relationships between TGF-β and glomerulosclerosis and interstitial fibrosis in aging must be considered cautiously until more extensive studies analyzing the complete TGF-β system are available.

One important question to be answered would be the cellular source of TGF-β in the present study. Circulating macrophages that infiltrate the kidney, as well as cells from the renal parenchyma (*i.e.*, tubular epithelial cells, interstitial fibroblasts), are a well known source of this cytokine in different experimental models of kidney disease (12). In the 24-mo-old rats included in the present experiments, the immunostaining results point to tubular cells as one of the possible sources for TGF-β. Other cell types, such as infiltrating macrophages in the case of experimental hydronephrosis (41), could be responsible for the increased TGF-β detected in old rats. However, the present



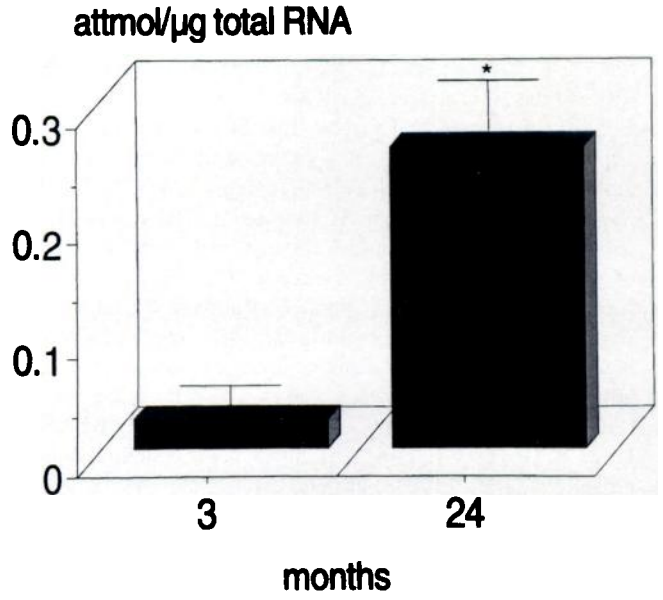
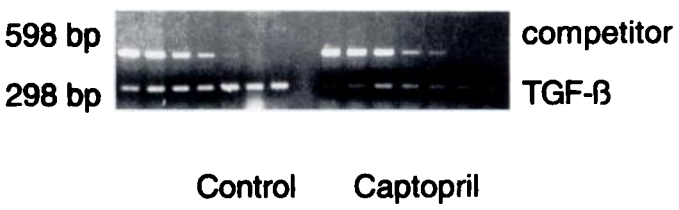


Figure 6. Quantitative measurement of TGF-β1 mRNA expression by RT-PCR in 3- and 24-mo-old rats. (Top) Characteristic agarose gel electrophoretic appearance of the simultaneous amplification products of the competitor and TGF-β1 in one representative rat from each group. Size of amplified products is indicated in base pairs (bp). (Bottom) Quantitative analysis of the amount of TGF-β1 mRNA, calculated as reported in Materials and Methods in the two groups of rats. Results are the mean ± SEM of 10 animals. $*P < 0.05$ *versus* 3 mo-old rats.



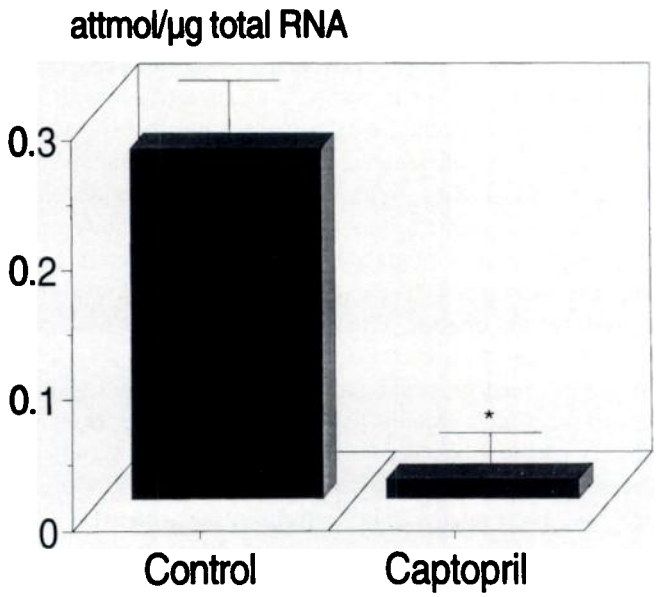


Figure 7. Quantitative measurement of the TGF-β1 mRNA expression by RT-PCR in 24-mo-old rats treated or not with captopril. (Top) Characteristic agarose gel electrophoretic appearance of the simultaneous amplification products of the competitor and TGF-β1 in one representative rat from each group. Size of amplified products is indicated in base pairs (bp). (Bottom) Quantitative analysis of the amount of TGF-β1 mRNA, calculated as reported in Materials and Methods in the two groups of rats. Results are the mean ± SEM of 10 animals. $*P < 0.05$ *versus* nontreated rats.

experiments were not devoted to analyzing this problem directly, and the degree of macrophage infiltration in the renal parenchyma was not measured. Therefore, the relative importance of the different cell types as the TGF-β source in aging cannot be concluded from the present results.

According to Gibbons *et al.* (42), angiotensin II induces TGF-β mRNA expression *in vitro*, and it has been demonstrated that the inhibition of the renin-angiotensin system can prevent TGF-β1 mRNA expression (2,10,37,43). Therefore, to clarify the importance of TGF-β1 in the development of different forms of tissue fibrosis in aging, we tested the long-term effects of captopril administration. Captopril treatment was associated with a decrease in protein excretion, but not with significant changes in systolic BP. In 24-mo-old rats, captopril significantly blunted the increase in TGF-β1 mRNA levels observed in nontreated animals of the same age, without modifying the degree of glomerulosclerosis, and significantly reducing interstitial fibrosis. This latter finding was recently reported in a model of aging in mice treated with enalapril, another angiotensin-converting enzyme inhibitor (44).

Together with the results of immunostaining, these findings support a role for TGF-β1 in the genesis of the morphologic changes in the kidney interstitium. Although it is well established that TGF-β1 expression underlies the development of chronic progressive tissue fibrosis in other experimental models of renal disease (2,8–11), this is the first time that TGF-β1 has been clearly linked to the development of interstitial fibrosis during aging. It is not apparent why, after captopril treatment, the decrease in TGF-β1 mRNA levels did not correlate with a reduction in TGF-β immunostaining in our histologic studies. The highly specific and sensitive competitive RT-PCR technique used might have revealed changes that cannot yet be detected by immunohistochemistry, considering that the antibody used also reacted with TGF-β2.

In contrast with our findings for interstitial fibrosis, we can

draw no definite conclusions regarding a hypothetical role of TGF-β1 in the development of glomerulosclerosis during aging, because other mechanisms may be involved. In aging rats, the severity of glomerular sclerosis correlates with plasma growth hormone levels (45). Interestingly, transgenic mice that chronically express growth hormone and insulin-like growth factor-1 develop progressive glomerulosclerosis (46). Age-related glomerulosclerosis may thus be dependent on pathogenic mechanisms different from those observed in interstitial fibrosis or other forms of progressive kidney fibrosis.

Unfortunately, the causes of TGF-β1 overexpression in aged rats are still incompletely understood. A genetically programmed modification in renal cells, with the subsequent changes in the extracellular matrix, is an attractive hypothesis. However, no experimental data support a mechanism of this type in aging. Environmental changes may also be involved; a more severe form of age-related glomerulosclerosis develops in rats fed a high-protein diet. Okuda *et al.* (47) showed that TGF-β expression is modulated by protein intake: A high-protein diet increases TGF-β expression, whereas a low protein diet produces the opposite effect. Although our rats were on a standard protein diet, it can be hypothesized that very long-term protein feeding could induce changes similar to those observed with high-protein diets. Certain oxygen species are highly reactive molecules, which may induce significant changes in renal physiology and morphology (48,49) and which have been related to other forms of age-related tissular changes. Results from our group have demonstrated an increased oxidative damage in kidneys from aged rats, suggesting that these molecules are another environment-related or genetically programmed factor involved in progressive age-associated modifications (50). However, at present no definite relationships have been established between reactive oxygen species and TGF-β1, but they should be explored.

In summary, we have reported the association between glomerulosclerosis and interstitial fibrosis and a progressive increase in TGF-β1 mRNA expression in aged rats. Immunostaining experiments and the findings after treatment with captopril suggest a cause-and-effect relationship between overexpression of this growth factor and increased interstitial fibrosis, with age-related glomerulosclerosis being a structural change apparently not related to TGF-β1. The mechanisms that trigger the overexpression of this growth factor in aging may differ from other forms of progressive renal disease, and should be explored in future experiments.

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