A ge-Dependent Expression of Fibrosis-Related Genes and Collagen Deposition in Rat Kidney Cortex

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Because progressive fibrosis is a histological hallmark of the aging kidney, we sought to characterize the course of some fibrosis-related genes [pro-α2(I)collagen (COL-I), pro-α1(III)collagen (COL-III), and transforming growth factors β1 and β3 (TGF-β1 and TGF-β3)] of interstitial collagen accumulation [COL-I and COL-III proteins, hydroxyproline (PRO-OH), histology] and its degradation (matrix metalloproteinase MMP-1 and -2) during maturation and early aging in rats. During the lifespan considered we observed no changes in the mRNA, except that COL-I mRNA tended to be up-regulated from 2 to 19 months of age. However, progressive fibrosis was histologically detectable, with COL-I accumulation (p < .05 and p < .01 in 12-month- and 19-month-old rats vs the youngest), and confirmed by the PRO-OH tissue levels (p = .001); COL-III seemed to be less involved. The MMP-1 protein level decreased significantly in the cortex of 12-month- and 19-month-old rats (p < .05), whereas MMP-2 protein level and activity remained essentially unchanged. These results show that, during aging of the kidney, (i) renal cortex fibrosis is explained by COL-I accumulation as a consequence of an altered balance between its synthesis and degradation, and (ii) the expression of the pleiotropic factor TGF-β in the renal cortex is not modified.

The process of aging involves progressive morphological and vascular alterations in the kidney concurrent with its functional decline (1,2). However, the mechanisms that account for this condition are not yet fully understood. As seen by light microscopy, accumulation of the main extracellular matrix (ECM) components leading to glomerulosclerosis and tubulointerstitial fibrosis is a hallmark of various chronic renal diseases (3), but is also frequently described in kidneys from aged animals and humans (4,5). Thus, scar formation is assumed to be a final common pathway, the end-stage lesions having similar composition. Matrix expansion appears to result from this excessive accumulation of ECM components and, above all, of the collagen proteins (4). This has been described for different organs, including the heart, for which the morphological and molecular aspects of the process have been characterized (6). Collagen content reflects the balance between formation and breakdown, with rapid turnover (7,8). Much of the newly synthesized collagen is immediately degraded; the extent of this degradation varies from one tissue to another but generally tends to decrease with age (9).

The rate of degradation of various matrix components is regulated primarily through the activity of matrix metalloproteinases (MMPs), which are also present in the glomeruli and proximal tubules (10). MMP activity is controlled at several levels in the cells, including the steps of synthesis and secretion, and inactivation by specific inhibitors (such as a matrix metalloproteinase inhibitor). The overall balance of collagen deposition can be affected by the transforming growth factor β (TGF-β) through a variety of biological activities: It directly increases the abundance and the stability of pro-α-collagen I and III transcripts, but also regulates the expression of several MMPs (11–13). For instance, elevated circulating levels of TGF-β1 are associated with collagen accumulation, and increased amounts of MMP-2 protein and mRNA for a MMP inhibitor (14). TGF-β1 also promotes the secretion of gelatinase from several cell types, including glomerular mesangial cells (15,16). However, little is known about its role in collagen deposition during renal aging (17,18).

Therefore a primary goal of the present study was to characterize the age-dependent course of the abundance of transcripts for type I and type III collagen and for TGF-β1 and TGF-β3. As a second aim, we correlated the quantitative expression of the mRNAs specific for these collagens with the amount of the relative proteins and of hydroxyproline (PRO-OH), a biochemical marker of total collagen accumulation. We also investigated whether metalloproteinase activity, particularly MMP-1 and MMP-2, was altered in relation to collagen accumulation during the development of age-related kidney fibrosis.

Materials and Methods

Animals

Twenty male Sprague–Dawley rats (Iffa Credo) were studied at 2, 6, 12, and 19 months of age (five animals per age group). On arrival, rats were individually housed for 15 days, with controlled temperature and light, then killed by decapitation. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with international policies (European

Quantification of mRNA Levels

After removal of the kidney, the cortex was separated from the medulla, immediately frozen in liquid nitrogen, and stored at −80°C, except for a portion cut out for histology. Total RNA was extracted from approximately 100 mg of frozen tissue by the acid guanidinium thiocyanate–phenol–chloroform method (19). RNA purity and concentration were determined spectrophotometrically. Total RNA was analyzed by northern blot to assess the specific hybridization with each cDNA probe, followed by slot hybridization assays for quantitative evaluation.

For northern blot, 20-μg samples were denatured and electrophoresed through 1% agarose gels. RNA integrity was verified by examination of the 28S and 18S ribosomal RNA bands of ethidium bromide stained gels under ultraviolet light. RNA was transferred to a nylon membrane (Gene Screen, New England Nuclear, Du Pont, Cologno Monzese-Milano, Italy) overnight by capillary blotting and backed at 80°C for 2 hours.

For slot blot analysis, total RNA (10, 4, 2 μg) was denatured in 50% dimethyl sulfoxide, 10-mM NaH₂PO₄ pH 7, 1M glyoxal for 1 hour at 50°C, applied directly to Z-probe membranes (Bio Rad) under gentle vacuum, and fixed at 80°C for 1 hour.

The cDNA probes for rat pro-α2(I)collagen (COL-I) (kindly provided by M. A. Zerm, MD, Thomas Jefferson University, Philadelphia, PA), rat pro-α1(III)collagen (COL-III) (kindly provided by Dr. E. Vuorio, University of Turku, Finland), and swine TGF-β1 and murine TGF-β3 (kindly provided by M. A. Zerm, MD, Thomas Jefferson University, Philadelphia, PA) were labelled with deoxyctydine 5′-[α-32P]-triphosphate (Amersham, Cologno Monzese-Milano, Italy) by primer extension (Random Primed DNA labeling kit, Boehringer Mannheim).

Membranes were prehybridized for 16–18 hours in a solution containing 50% formamide, 5× saline-sodium citrate buffer (SSC), 1% sodium dodecyl sulfate (SDS), 2× Denhardt’s reagent (50× reagent contains 5-g Ficoll, 5-g polyvinylpyrrolidone, and 5-g bovine serum albumin). Hybridization was done in the same solution, with 2 to 3 × 10⁶ cpm/ml of 32P-labeled cDNA added, for 16–18 hours at 42°C. Blots were then washed with 2× SSC at room temperature, 2× SSC 0.5% SSC at 65°C, 0.1× SSC at room temperature, 0.1× SSC 0.1% SDS 0.1% sodium pyrophosphate at 55°C, and exposed for 1–7 days at −80°C to autoradiographic films in cassettes with intensifying screens.

The filters were hybridized with a glyceraldehyde 3-phosphate dehydrogenase cDNA probe, a rat housekeeping gene, to normalize the results. Messenger RNA signals on autoradiographs were quantified by laser densitometry (IM1D, Amersham Pharmacia Biotech, Cologno Monzese-Milano, Italy).

Histology

Tissues for light microscopy were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, cut at four μm, and stained with hematoxylin–eosin and Masson trichrome.

Hydroxyproline

The frozen samples were homogenized and hydrolyzed in 6-N HCl for 24 hours at 110°C; PRO-OH was measured spectrophotometrically on the basis of its reaction with Ehrlich’s reagent, by the methods described by Stageman and Stalder (20).

Western Blotting

Renal cortex homogenates were prepared in 50-mM cool tris-HCl, pH 7.5, containing 0.1-M NaCl, 1-μg/ml leupeptin, 10-μg/ml aprotinin, and 2-M CaCl₂, and centrifuged for 5 minutes at 10,000 rpm. The supernatants were saved, and protein was determined by Lowry’s method (DC Protein Assay, Bio Rad, Segrate-Milano, Italy). The samples were prepared in a SDS sample buffer, boiled for 5 minutes, electrophoresed on 10% SDS polyacrylamide gel according to the procedure described by Laemmli (21), and electroblotted onto a nitrocellulose membrane by the Burnette method (22). To evaluate the collagen type III expression, 4-M urea was added to the gel (23).

After transfer, the blots were blocked for 1 hour with 5% skimmed milk in tris buffered saline with tween 20 (TBST), pH 8, then reacted for 1 hour with the first antibodies, rabbit–antisera collagen type I or III antiserum (Chemicon, Temecula, CA) diluted 1000-fold.

After reaction with the first antibody, the nitrocellulose membrane reacted with the second antibody; for collagen type I detection, a swine antirabbit IgG alkaline phosphatase-conjugated (Dako) serum was used at a 1:1000 dilution for 1 hour. The membrane was immediately washed three times in TBST, equilibrated for 10 minutes in an alkaline phosphatase buffer (100-mM tris-HCl, pH 9.5, 100-mM NaCl) and allowed to react with the substrate 5-bromo-4-chloro-3-indoxyl phosphate/p-nitroblue tetrazolium system (BCIP/NBT, Sigma).

For collagen type III, the signal was detected with a goat antirabbit peroxidase conjugated (1:1000) (Dako) serum and revealed by enhanced chemiluminescence (ECL western blotting reagent, Amersham Pharmacia Biotech). Blots were then exposed to autoradiography films.

Metalloproteinase Activity

MMP extraction.—Kidney cortex samples were homogenized in an ice-cold extraction buffer (1 ml/100 mg tissue) containing 10-mM cacodylic acid, 150-mM NaCl, 1-mM ZnCl₂, 20-mM CaCl₂, 1.5-mM NaN₃, and 0.01% Triton X-100 (pH 5). The homogenate was centrifuged (4°C, 5 minutes, 12,000 rpm) and the supernatant decanted and saved on ice. The final concentration of the kidney extracts was determined with a standardized colorimetric assay (DC Protein Assay, Bio Rad), and the samples were divided into aliquots and stored at −20°C. Each sample was run on SDS-polyacrylamide gel electrophoresis (PAGE) and stained by Coo massie blue to verify that the same amounts of total proteins were loaded in all lanes.

Zymography (MMP-2 activity).—Extracts were thawed on ice and mixed 3:1 with the substrate gel sample buffer (10% SDS, 4% sucrose, 0.25-M tris-HCl, pH 6.8, 0.1% bro-
mophenol blue). Each sample (30 μg) was loaded under nonreducing conditions onto electrophoretic minigel (SDSPAGE) containing 1 mg/ml of type I gelatin (Sigma, Milano, Italy). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), with a running buffer temperature of 4°C. After SDS-PAGE the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water and incubated overnight in a substrate buffer at 37°C (50-mM tris-HCl, 5-mM CaCl₂, 0.02% NaN₃, pH 8). After incubation the gels were stained in Coomassie blue, 30% methanol, 10% acetic acid, and destained in 30% methanol and 10% acetic acid. The lysis band areas were quantified by densitometric scanning (IM1D, Amersham Pharmacia Biotech).

MMP-1 and MMP-2 immunoblotting.—Renal extracts were diluted in SDS sample buffer, loaded on 10% SDS polyacrylamide gel, separated under reducing conditions at 40 mA according to Laemmli (21), and transferred at 100 V to a nitrocellulose membrane in 0.025-M tris-HCl, 192-mM glycine, 20% methanol, pH 8.3 (22). After electroblotting, the membranes were air dried and blocked for 1 hour. After being washed in PBST, membranes were incubated overnight at 4°C in polyclonal antibody to MMP-1 or MMP-2 (1:3000 dilution in PBST/bovine serum albumin 1%/NaN₃ 0.02%, Chemicon) and, after washing in horseradish peroxidase-conjugated goat antirabbit serum (1:10,000 dilution, Bio Rad) were scanned densitometrically.

Statistical Analysis

Statistical comparison between experimental groups was done with one-way analysis of variance (ANOVA). Because we were interested in relative changes more than in absolute differences, the raw data shown in the tables and figures were log transformed before the ANOVA was performed. Then, a post-test for a linear trend between age groups and Dunnett’s test (2-month age group taken as reference group) were carried out. Statistical analysis was performed with the GraphPad Prism version 3.0 software package (GraphPad Software, San Diego, CA). All results are expressed as mean ± standard error of the mean. A p value of < .05 was considered significant.

| Table 1. Rat Kidney Weights and Body Weights in Relation to Age |
|------------------|------------------|------------------|
| Age (months)     | Body Weight      | Kidney Weight    | Kidney Weight/Body Weight |
|                  | (g)              | (g)              | × 10^3                      |
| 2                | 314 ± 27         | 2.44 ± 0.16      | 8.01 ± 0.81                  |
| 6                | 541 ± 13*        | 3.65 ± 0.16*     | 6.75 ± 0.26                  |
| 12               | 614 ± 28*        | 3.49 ± 0.17*     | 5.78 ± 0.31**               |
| 19               | 768 ± 49*        | 4.23 ± 0.13*     | 5.56 ± 0.20*                |
| Analysis of variance (p value) | <.0001 | <.0001 | .0116 |

Note: Data are mean ± standard error of the mean for five animals per age group. *p < .01 vs 2-month, Dunnett’s post hoc test; **p < .05.

**Results**

Kidney Weight and Body Weight

The kidney weights (KWs) and body weights (BWs) of rats aged 2, 6, 12, and 19 months are presented in Table 1. KW increased with age but proportionally less than BW, so the ratio of KW to BW progressively declined from 2 to 12 months and remained constant thereafter.

Kidney Cortex Expression of Fibrosis-Related Genes

Figure 1 shows a representative northern blot for COL-I and glyceraldehyde 3-phosphate dehydrogenase messengers, and Figure 2 shows a slot blot for COL-III mRNA. The age-related changes in the abundance of COL-I and COL-III transcripts in kidney cortex are presented in Figure 3.

With aging there was no clear tendency for changes in COL-I (ANOVA, p = .228; trend analysis, p = .077) and COL-III (ANOVA, p = .140; trend analysis, p = .374) mRNA abundance. Aging did not significantly affect the levels of TGF-β1 (ANOVA, p = .590; trend analysis, p = .335).
but TGF-β3 mRNA was higher in the oldest animals: +98\% (p < .05 by Dunnett’s post-test) compared with 2-month-old rats (ANOVA, p = .038) (Figure 3).

Collagen Deposition in Kidney Cortex

**Histology.**—Light microscopy analysis of cortex sections from aged kidneys revealed that subtle focal structural changes start at the age of 6 months. Glomerular and tubular degeneration, atrophy, and scarring became more diffuse and marked at 12 and 19 months (Figure 4).

**Biochemical evaluation.**—Hydroxyproline content progressively increased with age (ANOVA, p = .006, Table 2) and was 91\% higher in 19-month-old than in 2-month-old rats (p < .001, Dunnett’s post hoc test).

**Western blotting.**—Figure 5 shows a representative western blot for COL-I. Quantification of the immunoreactive bands by densitometric analysis indicated an age-dependent accumulation of COL-I protein in the renal cortex (ANOVA, p = .001) with a 43\% (p < .05, Dunnett’s post hoc test) and a 91\% (p < .01) increase compared with that of a 2-month-old rat [Figure 6(a)]. By contrast, COL-III levels tended to decrease throughout the study period (ANOVA, p = .056; linear trend, p = .032) [Figure 6(b)].

Collagen Degradation

Immunoreactive MMP-1 levels in the renal cortex decreased with aging (ANOVA, p = .088; linear trend, p = .018), with a 35\% and a 34\% reduction at 12 and 19 months, respectively, compared with those of 2-month-old animals (Dunnett’s post hoc test: p < .05 for both age groups) (Fig-
Figure 7). Although MMP-2 protein levels were not altered during the study period [ANOVA, \( p = .840 \), Figure 8(a)], gelatinase activity [Figure 8(b)] tended to increase (ANOVA, \( p = .0295 \); linear trend, \( p = .033 \)).

**Discussion**

Kidney growth in the rat is strain and possibly also supplier dependent. In the present study, Sprague–Dawley rats showed continuous body growth between 2 and 19 months of age, with a reduction of the KW to BW ratio between 2 and 12 months but no change thereafter. These observations are in good agreement with those of Schaefer and colleagues (24), who reported similar increments in kidney mass of Wistar rats.

The KW increase is generally related to expansion of the ECM components; glomerulosclerosis and tubulointerstitial fibrosis are histological hallmarks of progressive renal diseases of different etiologies but are also described in kidneys from aged humans and animals. Because fibrosis impairs renal function and plays a pivotal role in progression to chronic failure, better knowledge of the basic mechanisms would be extremely useful.

The composition of the renal ECM and its age-related changes have been investigated by immunofluorescence microscopy (4). However, to the best of our knowledge, such analysis has never considered in the same experimental model the molecular and biochemical mechanisms that control the turnover of collagen proteins.

![Figure 4. Microphotographs showing sections of kidney cortex, stained with Masson trichrome, representative of rats aged A, 2, B, 6, C, 12, and D, 19 months. Original magnification: 250×.](image)

![Figure 5. Western blot analysis of COL-I [pro-α2(I) collagen] protein levels in kidney cortex of 2-, 6-, 12-, and 19-month-old rats. Immunoreactive bands were obtained with an anti-COL-I antibody as described in the Material and Methods section. Each lane refers to one animal.](image)

### Table 2. Rat Kidney Cortex Hydroxyproline Content in Relation to Age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Hydroxyproline (μg/mg tissue)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>0.242 ± 0.002</td>
</tr>
<tr>
<td>6</td>
<td>0.262 ± 0.022</td>
</tr>
<tr>
<td>12</td>
<td>0.312 ± 0.021</td>
</tr>
<tr>
<td>19</td>
<td>0.464 ± 0.010*</td>
</tr>
</tbody>
</table>

*Note: Data are mean ± standard error of the mean for five animals per age group.

*\( p < .001 \) vs 2-month-old rats, Dunnett’s post hoc test.
Light microscopy analysis of our histological samples confirmed the progressive fibrosis in kidney cortex and the tubulointerstitial accumulation of collagen during aging. This process was reflected in biochemical terms by the progressive increase in the content of PRO-OH and COL-I proteins over the life span considered.

The tendency to up-regulation of COL-I mRNA transcript levels from 2 to 19 months is synchronous with COL-I deposition in the kidney cortex and thus suggests a transcriptional mechanism of regulation. On the other hand, COL-III messenger expression and the immunoreactive level tended to decrease in the aging renal cortex, an observation, that is in line with the hypothesis of its progressive replacement with COL-I (25,26).

Because there is experimental evidence suggesting a key role for TGF-β1 in the control of renal fibrosis in various diseases (27–33), we decided to measure the expression of this factor in the aging renal cortex. We found that TGF-β1 mRNA abundance remained unchanged between 2 and 19 months of age, with a nonsignificant tendency to decrease, whereas TGF-β3 mRNA levels tended to be higher in the oldest animals (p < .05). Therefore, in our study, collagen deposition seemed to be unrelated to TGF-β1 gene expression. This suggests that the control exerted by TGF-β on collagen deposition during the progressive renal fibrosis associated with natural aging is different from its role in most kidney diseases. The same situation has been described for the heart, in which the age-related accumulation of interstitial and perivascular collagen was not accompanied by changes in TGF-β1 and TGF-β3 expressions (6) and also in accordance with a previous study on the kidney (17). However, our results contrast with a recent report in which fibrosis of the renal cortex of aged Wistar rats was associated with increased levels of both TGF-β1 mRNA and protein (18). Thus the involvement of TGF-β1 in the progression of age-related renal fibrosis cannot be excluded because of posttranscriptional regulation, the obligatory activation of the secreted latent form of the cytokine, and its interaction
with cell receptors, all steps capable of controlling the fibrogenic activity of this growth factor's family.

Enhanced collagen deposition in the kidney of old rats may also result from slower breakdown. Collagen degradation is catalyzed by proteolytic enzymes, present in glomeruli and tubular epithelial cells (10). The metalloproteinases with collagenase and gelatinase activities are secreted in the extracellular space and are activated by proteolytic cleavage after densitometric analysis. Data are means ± standard errors of the mean for five animals per age group.

Figure 8. Matrix metalloproteinase-2 (MMP-2) in the kidney cortex of aging rats; each lane refers to a single animal. A, Immunoblotting: the antibody identifies a positive immunoreactive band in the 66/72-kDa region corresponding to MMP-2. B, Representative gelatin zymogram of gelatinase activity; the lytic activity in the 66/72-kDa region is consistent with MMP-2. C, A bundance of gelatinase activity after densitometric analysis. Data are means ± standard errors of the mean for five animals per age group.

because of an altered balance between synthesis and degradation. These results are consistent with a previous report (24) of significantly reduced collagenase activity with advancing age. Although there was no age-dependent change in MMP-2 levels, gelatinase activity was increased, in contrast to an observation made in 20-month-old Munich Wistar rats (38). However, data about gelatinase levels in the aging kidney are discordant and may depend on the rat strain.

Whether reduced renal proteinase activities reflect a decrease in intracellular and extracellular protein degradation is still debated. The fact that the MMP-2 immunoreactive level was not altered during aging is not in contradiction to the accumulation of interstitial collagen in the senescent kidney cortex as this metalloproteinase is not active on intact collagen (15,36).

Another possibility is that protein degradation in aged rat kidneys may be slower because of some change in the activity of the enzymes that regulate the collagen cross-linking level, which would shift the ratio of labile to highly cross-linked collagen toward the latter (39,40), thus making the protein less susceptible to the action of proteases.

These findings as a whole suggest that altered protein turnover may be a cause of age-related collagen accumulation, particularly glomerulosclerosis and tubulointerstitial fibrosis.

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References


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