Development of age-dependent glomerular lesions in galectin-3/AGE-receptor-3 knockout mice

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Iacobini, Carla, Giovanna Oddi, Stefano Menini, Lorena Amadio, Carlo Ricci, Clelia Di Pippo, Mariella Sorcini, Flavia Pricci, Francesco Pugliese, and Giuseppe Pugliese. Development of age-dependent glomerular lesions in galectin-3/AGE-receptor-3 knockout mice. Am J Physiol Renal Physiol 289: F611–F621, 2005. First published May 3, 2005; doi:10.1152/ajprenal.00435.2004.—Aging is a complex pathophysiological condition in which the function of many organ systems becomes altered, although it is unclear the extent to which these changes are the result of a normal aging process or of the interplay of age with chronic diseases that are more common in older people. Several changes in kidney function and structure have been detected in aged humans (31) and animals (4). The functional hallmark is a progressive decline of glomerular filtration rate, associated with reduced renal plasma flow and ultrafiltration coefficient, and increased filtration fraction and renal vascular resistance (19, 31). From a morphological point of view, the glomerular number is reduced (36), and there is an increased prevalence of global glomerulosclerosis and tubulointerstitial fibrosis, preceded and accompanied by glomerular hypertrophy, mesangial matrix expansion, glomerular basement membrane thickening, and arteriolar hyalinosis (4, 31). These structural changes resemble those detected in diabetes (18).

Also, aging shares with diabetes some biochemical abnormalities that might play a major role in the pathogenesis of vascular and renal changes occurring in both conditions. One popular theory proposes that the characteristic loss of elasticity of tissues of aged individuals is dependent on the progressive accumulation of advanced glycation end-products (AGEs; see Ref. 49). An AGE cross-link breaker was shown to reverse age-related increases in myocardial and arterial stiffness (2, 54). The aging process has also been related to cumulative oxidative damage caused by reactive oxygen species (ROS) over a lifetime (28). In both invertebrates and mammals, increased longevity correlates with enhanced resistance to oxidative stress (12), and deletion of p66shc, a protein involved in ROS-induced apoptosis, was shown to be associated with increased resistance to oxidative stress and increased life span (35).

These biochemical abnormalities are strictly related to each other. In fact, AGEs are heterogeneous compounds that accumulate in tissues due to increased formation/intake and reduced degradation/excretion; AGE formation occurs through both nonoxidative and oxidative reactions (11). Enhanced mitochondrial superoxide production, resulting from excess glucose disposal and causing diversion of glucose flux toward the AGE-precursor methylglyoxal via inhibition of glycolaldehyde phosphate dehydrogenase, is now considered as the main mechanism of AGE formation in diabetes (7). Aging is also characterized by less efficient electron transport in the mitochondrial respiratory chain, with consequent increase in superoxide production, and calorie restriction, an intervention known to prolong life span, was found to proportionately decrease mitochondrial ROS generation, especially at complex I (3, 50). AGEs exert both direct (physicochemical) and indirect (biological) effects, the latter mediated by cell surface receptors. AGE receptors have a dual function, since they are involved in AGE removal, and also in AGE-induced cell activation (52), via redox-sensitive signaling pathways triggered by AGE receptor-mediated generation of ROS (56). These pathways lead to p21ras/mitogen-activated protein kinase (MAPK)-dependent activation of transcription factors such as...
The working hypothesis was that galectin-3 KO mice develop levels and oxidative stress characterizing the aging process. Galectin-3 KO mice and their relation to the increased AGE disease by assessing the development of glomerular lesions in vivo to afford protection toward AGE-induced tissue injury, as providing evidence that galectin-3 plays a significant role intributable to the lack of galectin-3 AGE receptor function, thus

The numerous AGE-binding proteins include the receptor for AGEs (RAGE; see Ref. 47), mainly involved in cell activation, the macrophage scavenger receptors (MSRs) class A (20) and B (38), which participate in AGE removal, and OST-48/AGE-receptor (R) 1 (33), 80K-H/AGE-R2 (33), and galectin-3/AGE-R3 (51), thought to behave as an AGE receptor complex.

Galectin-3 consists of a short NH2-terminal domain containing a unique proline-glycine-alanine-thyrosine-rich repeat motif and a highly conserved COOH-terminus containing the carbohydrate recognition domain (CRD; see Ref. 41). These unique structural properties and the dual localization of galectin-3 determine two different modes of interaction with proteins (via its NH2-terminus and CRD, respectively) and enable it to exert multiple functions (40). Intracellularly, galectin-3 acts as a pre-mRNA splicing factor (10) and also regulates the cell cycle, with promotion of replication and inhibition of apoptosis (58). Extracellularly, galectin-3 regulates cell adhesion in a dual manner: it promotes hom- and heterotypic cell-to-cell interactions (24), whereas it downregulates cell adhesion to the ECM (37). Extracellular galectin-3 is also capable of interaction with IgE and the IgE receptor, which induces mast cell activation (13) and the high-affinity binding, internalization, and degradation of AGEs (51, 60).

Recently, we have reported that galectin-3 is not or is weakly expressed at the glomerular/mesangial level under normal conditions and is induced/upregulated in diabetes (43) and that galectin-3 knockout (KO) mice are more prone to develop glomerular disease induced by diabetes and AGE injection compared with the corresponding wild-type (WT) animals (23, 42). The more marked increases in circulating and renal tissue AGE levels in the diabetic (and AGE-injected) KO vs. WT mice and the altered AGE receptor expression pattern in the galectin-3-deficient genotype suggested that it was attributable to the lack of galectin-3 AGE receptor function, thus providing evidence that galectin-3 plays a significant role in vivo to afford protection toward AGE-induced tissue injury, as opposed to RAGE (22).

This study was aimed at investigating the role of the AGE/AGE receptor pathway in the pathogenesis of age-related renal disease by assessing the development of glomerular lesions in galectin-3 KO mice and their relation to the increased AGE levels and oxidative stress characterizing the aging process. The working hypothesis was that galectin-3 KO mice develop accelerated age-related renal disease compared with coeval WT mice because of their increased susceptibility to AGE-induced injury.

METHODS

Experimental design. Male galectin-3 KO mice, generated by gene ablation (21), and the corresponding C57BL6 WT mice were studied at 2, 6, 12, 18, and 24 mo of age (n = 6–8 animals/group). The animals were housed and cared for in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85–23, revised 1985) and national laws and had free access to food and water. Before death, mice were placed in metabolic cages to collect urine samples for total protein, albumin, and creatinine measurements. Next, the animals were weighed and anesthetized with intraperitoneal ketamine (Imalgene, 60 mg/kg body wt) and xylazine (Rompun, 7.5 mg/kg body wt), a laparotomy was performed, and a blood sample was obtained for measurement of blood glucose, serum creatinine and AGE levels, and plasma isoprostane 8-epi-prostaglandin PGF2α concentrations. Finally, both kidneys were quickly removed, cleaned of surrounding fat, washed in sterile saline solution, and weighed. A sagittal section of the right kidney was immediately fixed by immersion in phosphate-buffered 4% formaldehyde solution and processed for light microscopic examination and morphometric evaluation, as previously described (23, 42). Paraffin-embedded sections were used also for immunohistochemistry for fibronectin and collagen IV, to assess the extent of renal ECM accumulation, as well as Nα-carboxymethyllysine (CML) and 4-hydroxy-2-nonenal (HNE), to assess the extent of glycoxidation and lipoxidation reactions, respectively (5), and the distribution of these products within the kidney tissue. The remaining tissue from the right kidney was frozen in liquid nitrogen and subsequently used for either total or nuclear protein extraction, as previously described (23). Total protein extracts were used for the assessment of fibronectin and collagen IV protein expression and AGE content, whereas nuclear extracts were used for measurement of NF-κB activity. The renal cortex from the left kidney was separated from medulla and used for total RNA extraction by the guanidine thiocyanate-phenol-chloroform method using TRizol Reagent (Invitrogen Italia, San Giuliano Milanese, Italy). The kidney cortex mRNA levels of the following targets were then assessed: the ECM components fibronectin, laminin B1, and collagen IV α1-chain; the profibrogenic cytokine transforming growth factor (TGF)-β1; and the AGE receptors OST-48/AGE-R1, 80K-H/AGE-R2, galectin-3/AGE-R3, RAGE, and MSR-A type II.

Renal function. Serum creatinine levels were measured by the Jaffé method (23, 42). Total proteinuria was assessed by the Bradford method using a Bradford dye-binding protein assay kit (Pierce Chemical, Rockford, IL), whereas albuminuria was measured with a Mouse Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX); both values were normalized by urine creatinine concentration (ng/mg). Nuclear factor (NF)-κB activity. The renal cortex from the left kidney was separated and used for total RNA extraction by the guanidine thiocyanate-phenol-chloroform method using TRizol Reagent (Invitrogen Italia, San Giuliano Milanese, Italy). The kidney cortex mRNA levels of the following targets were then assessed: the ECM components fibronectin, laminin B1, and collagen IV α1-chain; the profibrogenic cytokine transforming growth factor (TGF)-β1; and the AGE receptors OST-48/AGE-R1, 80K-H/AGE-R2, galectin-3/AGE-R3, RAGE, and MSR-A type II.

Renal structure. Analysis of renal structure was performed by two pathologists blinded to the group assignment of the specimens on multiple 4-μm kidney tissue sections stained with periodic acid-Schiff (PAS) or Alcian blue. Sections were evaluated for total protein, albumin, and creatinine measurements. Next, the mean mesangial area (mMA) was calculated by the formula: (N1 × N2 × N3 × N4 × C)/100, where N1, N2, N3, and N4 represent the numbers of glomeruli exhibiting grades 1, 2, 3, and 4, respectively, and C is the number of glomeruli assessed (i.e., 100). Morphometric analysis was performed by the use of a custom-made, C-language macro written with the Optimas 6.5 image analysis system (Optimas; MediaCybernetics, Silver Spring, MD), as previously reported in detail (23). Briefly, the areas of at least 60 glomerular tuft profiles/sample were measured, the harmonic mean of the profile area (mean glomerular area, mGA) was obtained, and the mean glomerular volume (mGV) was estimated from it. Next, periodic acid-Schiff (PAS)-positive material in each of these glomeruli was quantified and expressed as the percentage of the glomerular tuft area (fractional mesangial area, fMA). The color threshold was set by identifying three to five separate pixels in areas of positive staining. Finally, the mean mesangial area (mMA) was calculated by the formula (FMA × mGA)/100.

Renal ECM, TGF-β1 and AGE receptor gene expression. Transcripts for fibronectin, laminin B1, collagen IV α1-chain, TGF-β1, AGE-R1, AGE-R2, galectin-3/AGE-R3, RAGE, and MSR-A type II...
were quantified by competitive RT-PCR (23, 42). Total RNA (1 μg) was reverse transcribed using a Retroscript kit (Ambion, Austin, TX). The following primers were used for fibronectin: 5′-AGG GTG TGC TAC TCT GT-3′ and antisense 5′-GAT GCA ATC TCG GAG AC-3′; laminin B1 sense 5′-CAA GCT TGA GAG AGG AAC AAG G-3′ and antisense 5′-TGA CTT TGG TCA CCG AGC-3′; collagen IV α1-chain sense 5′-TCT GAC GCT CCT GGT TAT AAG TTA GGC TTT CGA TTC AGC-3′ and antisense 5′-CAG G-3′; AGE-R1 sense 5′-GGT GCC CCT TCA CCT TCT CCT TCT CTC CTT-3′ and antisense 5′-GCT CTT CCA CTC CTC CTT ACT CCT-3′; AGT GCC AGG CAA GGA TTA CCT TGG TCA CCG AGT GTG AGG TCT ATG TC-3′ and antisense 5′-GCA CGG AGG CAA CTA TGA AC-3′; AGE-R2 sense 5′-TGG TGT GCC GTC TTA CCT TGG C-3′ and antisense 5′-CGG AGT AGT CTT CCT TGA CCT GAT TCT AC-3′; antisense 5′-GACA CAG GTC AAG GTC-3′ and antisense 5′-GCA CAC CTG CAC CTG GAG TCT AC-3′; and antisense 5′-GGG AAG CT-3′. Competitive PCR was performed by using increasing amounts of mutants made by creating a deletion in the original PCR product, and preliminary experiments were performed to establish the range of mutant concentrations producing a slope of the line close to one and within which the equivalence point falls. After electrophoresis of PCR products, the ratio of unknown cDNA/mutant was quantified by scanning densitometry using ImageJ software, a public domain Java image processing program inspired by NIH Image, and results were expressed as the ratio of each target to β-actin mRNA level.

**Renal ECM protein expression.** Protein expression for fibronectin and collagen IV was assessed by immunohistochemistry (23). Briefly, deparaffinized kidney sections were microwaved two times for 3 min in Reveal Heat Mediated Antigen Retrieval Solution, pH 6.0 (Abcam, Cambridge, UK), and endogenous peroxidase activity was quenched by incubating sections in 2% (vol/vol) hydrogen peroxide in PBS for 30 min, whereas nonspecific binding was blocked by incubation in 10% normal goat serum (Sigma) for 45 min at room temperature. Next, sections were incubated overnight at 4°C with affinity-purified rabbit anti-human polyclonal antibody (Sigma) and the rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1452–1618 mapping at the COOH terminus of human collagen-α1 type IV (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:400 and 1:200, respectively, in PBS containing 1% normal goat serum. Sections were then incubated for 1 h at room temperature with 1:300 diluted biotinylated goat anti-rabbit antibody (Dako), followed by 30-min incubation with streptavidin-biotinylated horseradish peroxidase complex (StreptABComplex/HRP; Dako). Finally, sections were developed with 3,3′-diaminobenzidine in chromogen solution (Liquid DAB+; Dako) for 3 min and counterstained with hematoxylin for 30 s. In the negative controls, the primary antibody was omitted. The sections were analyzed using the Optimas 6.5 image analysis system, and the results were expressed as percent glomerular area that was positive to each product.

**Renal NF-κB activity.** The activity of NF-κB p65 was assessed by ELISA using a TransAMNF-p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA; see Ref. 23). This method is based upon reaction of nuclear extracts (30 μg protein) with specific oligonucleotide sequences coated on a microtiter plate. According to the manufacturer’s instructions, the binding of activated NF-κB was revealed by the addition of a primary polyclonal anti-NF-κB p65 antibody, followed by a secondary antibody conjugated with horseradish peroxidase and the 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was finally read at 450 nm, and after the reaction was stopped with sulfuric acid, 655 nm using a microtiter plate reader.

**Statistical analysis.** Values are expressed as means ± SD; the percent change in KO vs. WT animals was also calculated. Statistical significance was evaluated by Student’s t-test for comparison between KO and WT mice at each age and one-way ANOVA for comparison among different age groups. Correlation between serum or kidney tissue AGE levels and parameters of renal function and structure were also calculated using linear regression analysis. All statistical tests were performed on raw data.

**RESULTS**

**Body weights.** Significant body growth occurred only from 2 to 6 mo of age and, to a lesser extent, from 6 to 12 mo. Thereafter, body weight remained stable, with no significant difference between the two genotypes at any age (Table 1).

**Renal function.** Serum creatinine levels increased only slightly (by ~10%) from 2 to 24 mo of age, with no significant difference between KO and WT mice (Table 1). Urinary protein/creatinine and albumin/creatinine ratios increased with age in both genotypes, with significantly higher levels in KO vs. coeval WT mice at 18 (by 31 and 49%, respectively) and 24 (by 55 and 65%, respectively) mo of age (Fig. 1, A and B).

**Renal structure.** Aged WT mice showed only a modest degree of glomerular and tubulointerstitial damage; conversely, significant renal lesions were detectable in KO mice, particularly at 24 mo of age (Fig. 2). There were PAS-positive deposits within the mesangium, thickening of glomerular basement membrane and Bowman’s capsule (with formation of synechiae and capsular fibrosis), extensive glomerular sclerosis (prevailing at the vascular pole), iainosis of the afferent arterioles, proteinaceous and hyaline casts in the distal tubuli, and vacuolar degeneration of the cortical tubuli with interstitial fibrosis. All structural parameters increased significantly with
Table 1. Final body weight, serum creatinine, kidney wet weight, GSI, and circulating and renal AGE levels in galectin-3 KO vs. WT mice of 2, 6, 12, 18, and 24 mo of age

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Genotype</th>
<th>Final body weight, g</th>
<th>Final creatinine, mg/dl</th>
<th>Kidney wet weight, g</th>
<th>GSI</th>
<th>Circulating AGE levels, µg/ml</th>
<th>Renal AGE levels, µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mo</td>
<td>WT</td>
<td>21.1 ± 1.3 (6)</td>
<td>0.9 ± 0.1 (6)</td>
<td>20.5 ± 1.5 (6)</td>
<td>0.18 (6)</td>
<td>3.0 ± 0.2 (6)</td>
<td>0.1 ± 0.01 (6)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>21.4 ± 1.7 (6)</td>
<td>1.3 ± 0.7 (6)</td>
<td>20.8 ± 1.5 (6)</td>
<td>0.18 (6)</td>
<td>3.5 ± 0.3 (6)</td>
<td>0.2 ± 0.01 (6)</td>
</tr>
<tr>
<td>6 mo</td>
<td>WT</td>
<td>27.2 ± 2.8 (8)</td>
<td>1.3 ± 0.7 (6)</td>
<td>22.0 ± 2.0 (8)</td>
<td>0.21 (8)</td>
<td>4.0 ± 0.4 (8)</td>
<td>0.4 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>28.5 ± 2.8 (8)</td>
<td>1.4 ± 0.7 (6)</td>
<td>22.5 ± 2.0 (8)</td>
<td>0.21 (8)</td>
<td>4.6 ± 0.5 (8)</td>
<td>0.5 ± 0.01 (8)</td>
</tr>
<tr>
<td>12 mo</td>
<td>WT</td>
<td>35.5 ± 1.8 (6)</td>
<td>1.3 ± 0.7 (6)</td>
<td>24.5 ± 1.6 (6)</td>
<td>0.21 (8)</td>
<td>5.4 ± 0.6 (8)</td>
<td>0.6 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>36.2 ± 1.8 (6)</td>
<td>1.5 ± 0.7 (6)</td>
<td>25.2 ± 1.6 (6)</td>
<td>0.21 (8)</td>
<td>6.0 ± 0.7 (8)</td>
<td>0.7 ± 0.01 (8)</td>
</tr>
<tr>
<td>18 mo</td>
<td>WT</td>
<td>34.5 ± 1.6 (6)</td>
<td>1.5 ± 0.7 (6)</td>
<td>24.0 ± 1.6 (6)</td>
<td>0.21 (8)</td>
<td>5.3 ± 0.6 (8)</td>
<td>0.6 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>35.2 ± 1.6 (6)</td>
<td>1.8 ± 0.8 (6)</td>
<td>25.0 ± 1.6 (6)</td>
<td>0.21 (8)</td>
<td>6.0 ± 0.7 (8)</td>
<td>0.7 ± 0.01 (8)</td>
</tr>
<tr>
<td>24 mo</td>
<td>WT</td>
<td>34.0 ± 1.8 (6)</td>
<td>1.5 ± 0.7 (6)</td>
<td>23.5 ± 1.8 (6)</td>
<td>0.21 (8)</td>
<td>5.0 ± 0.6 (8)</td>
<td>0.6 ± 0.01 (8)</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>34.9 ± 1.8 (6)</td>
<td>1.8 ± 0.8 (6)</td>
<td>24.8 ± 1.8 (6)</td>
<td>0.21 (8)</td>
<td>6.0 ± 0.7 (8)</td>
<td>0.7 ± 0.01 (8)</td>
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Values are means ± SE, n = number in parentheses. WT, wild-type KO breeders; GSI, glomerular sclerosis index. AGE, advanced glycation and protein. Significance different from the coeval WT mice (Table 1 and Fig. 1, C–E).

Renal ECM and TGF-β1 expression. ECM and TGF-β mRNA expression increased significantly with age in both genotypes. However, the age-dependent increases were more pronounced in the KO mice (+34–48% at 24 vs. 2 mo of age) than in the WT mice (+31–34%). Transcript levels were significantly higher in KO vs. WT animals starting at 12 mo of age, with maximal increases detected at 24 mo of age (fibronectin +38%, laminin B1 +46%, collagen IV α1-chain +53%, and TGF-β1 +25%; Fig. 3). Fibronectin and collagen IV protein expression at the renal/glomerular level also increased with age in both genotypes and was significantly more pronounced in KO than WT mice, particularly at 24 mo of age (Fig. 4).

Circulating and renal tissue AGE levels. Circulating and renal tissue AGE levels increased significantly with age in both genotypes, although increases were more marked in KO vs. WT mice, with differences that were significant at 12 (+26 and 31%, respectively), 18 (+32 and 43%), and, particularly, 24 (+46 and 55%) mo of age (Table 1). Both serum and kidney AGE levels correlated significantly with proteinuria, GSI, and fMA (Fig. 5) as well as with other parameters of renal function and structure, including albuminuria ($R^2 = 0.83, P = 0.000001$ for both), mGA ($R^2 = 0.45$ and 0.64, $P = 0.004$ and 0.0002, respectively), and mMA ($R^2 = 0.79$ and 0.85, $P = 0.000005$ and 0.0000004, respectively).

Renal AGE receptor gene expression. The kidney cortex gene expression for AGE receptors showed small variations with age in the WT mice, with reduction of OST-48/AGE-R1 (−19%) and increase in 80K-H/AGE-R2 (+21%), galectin-3/AGE-R3 (+28%), RAGE (+22%), and MSR-A type II (+20%). A more marked age-dependent increase in RAGE (+30%) together with similar changes in OST-48/AGE-R1, 80K-H/AGE-R2, and MSR-A type II (+17%), was detected in the KO mice. Transcripts for 80K-H/AGE-R2 and RAGE were significantly higher at any age (+42–45% and 32–38%, respectively), and those for MSR-A type II were significantly lower starting at 12 mo (−12 to 14%) in KO vs. WT mice (Fig. 6), whereas no significant difference in the mRNA level for OST-48/AGE-R1 was observed between the two genotypes.

Plasma isoprostane 8-epi-PGF$_2\alpha$ levels. Plasma levels of isoprostane 8-epi-PGF$_2\alpha$ increased with aging in both genotypes. However, increases were significantly more marked in KO than in WT mice; values were 152.2 ± 14.4 pg/ml at 24 mo vs. 93.4 ± 6.3 at 6 mo (+63%, $P < 0.001$) in KO and 126.5 ± 16.5 vs. 91.2 ± 9.5, respectively (+38%, $P < 0.01$), in the WT animals.

Renal CML and HNE levels. Renal content of CML and HNE was increased in KO vs. WT mice at 18 and, particularly, 24 mo of age (Fig. 7). In the glomeruli, CML was only barely detectable in KO, but not WT mice, whereas HNE immunoreactivity was more pronounced than that of CML and was age in both genotypes. Although the kinetics of age-related changes in kidney structure were similar in the two groups, the amplitude was much higher in KO than WT mice. As a consequence, kidney wet weight (+8%) at 24 mo of age, GSI (+31–74%), mGA (+12–21%), and mGV (+18–33%) starting at 18 mo, and IMa (+18–21%) and mMA (+21–46%) starting at 12 mo of age, were significantly higher in KO vs. coeval WT mice (Table 1 and Fig. 1, C–E).
significantly higher in KO vs. WT animals (19.73 ± 3.02 vs. 8.751 ± 2.57% of glomerular area, \( P < 0.001 \)).

\textit{Renal NF-κB activity.} The activity of NF-κB p65 within the kidney tissue increased significantly in aged mice from both genotypes. However, transcription factor activity increased more markedly in KO (0.26 ± 0.04 OD at 24 mo vs. 0.17 ± 0.03 at 6 mo, +54%, \( P < 0.005 \)) than in WT (0.21 ± 0.03 vs. 0.16 ± 0.03, respectively, +31%, \( P < 0.05 \)) mice.

Fig. 2. Histological appearance of kidney sections from WT and galectin-3 KO mice of 12 (A and B) and 24 (C and D) mo of age [periodic acid-Schiff (PAS), original magnification \( \times 400 \)]. PAS highlights extracellular matrix (ECM) deposits (bright pink stain) in the mesangium (arrows) and thickness of Bowman’s capsule (triangles), which were more pronounced in KO than WT mice. As a result of mesangial expansion, capillary lumen become compromised in KO mice, with capillary occlusion and aneurysm formation (*).
DISCUSSION

In these studies, we could extensively characterize the renal functional, structural, biochemical, and molecular changes occurring with age in normal mice and detect similarities and differences with those observed in diabetic (and AGE-injected) animals. Aging mice exhibited progressive increases in total proteinuria, albuminuria, GSI, glomerular area and volume, fractional and absolute mesangial area, circulating and renal tissue AGE and plasma isoprostane 8-epi-PGF2α levels, kidney CML and HNE content, and renal NF-kB p65 activity. In addition, they showed an age-dependent increase in kidney cortex mRNA level for the ECM proteins fibronectin, laminin, and collagen IV, the prosclerotic cytokine TGF-β1, and the AGE receptors 80K-H/AGE-R2, galectin-3/AGE-R3, RAGE, and MSR-A type II, with reduction of OST-48/AGE-R1 gene expression. Fibronectin and collagen IV protein expression at the renal/glomerular level also increased with age.

The results of our study are in keeping with previous reports in experimental animals, also showing the development of proteinuria, glomerular sclerosis, and tubulointerstitial fibrosis with increasing age (4). In particular, our data are consistent with previous studies in >2-yr-old rats, showing a progressive accumulation of ECM proteins (1) and TGF-β1 (44) and an upregulation of their mRNA levels, indicating an increased matrix synthesis (9). However, the extent of ECM and TGF-β1 mRNA upregulation in our study was lower than that detected in aged rats and closer to that previously observed in diabetic animals, also showing the development of changes in renal function and structure, associated with more marked increases of AGE levels, in keeping with the absence of large age-related variations in mRNA expression in rat kidneys (40). Our results are also in agreement with earlier reports showing age-dependent accumulation of CML (45) and HNE (25) and increases in plasma isoprostane 8-epi-PGF2α levels (39). Finally, the increase in renal NF-kB p65 activity is in keeping with previous data from Fischer 344 rats (27) and also with reports from other tissues of aged animals, including liver (17), brain (30), heart (16), and gastric mucosa (55).

These changes closely resemble those previously detected by our group in mice rendered diabetic with streptozotocin or injected with CML (42, 23), although the extent of increases in serum and kidney AGE levels, renal CML and HNE accumulation, and NF-kB p65 activity was lower in aged mice (also the degree of proteinuria was less). On the other hand, these biochemical abnormalities lasted for a longer period of time than in diabetic and CML-treated mice and paralleled closely the development of changes in renal function and structure, which occurred more slowly since they were fully expressed only at 18–24 mo of age.

We could also confirm the working hypothesis that galectin-3 KO mice are more prone to develop age-dependent renal disease than the WT animals, in keeping with previous reports from our group showing accelerated glomerulopathy in diabetic or AGE-injected galectin-3 KO animals vs. their corresponding WT controls (42, 23). This was indicated by the finding that KO mice had more pronounced changes in renal function and structure, associated with more marked increases in circulating and renal tissue AGE levels, plasma isoprostane 8-epi-PGF2α concentrations, renal content of the glycoxidation and lipoxidation products CML and HNE, and renal NF-kB p65 activity compared with coeval WT mice. KO mice also showed a higher transcript level (and more pronounced age-dependent increases) of 80K-H/AGE-R2 and RAGE, together with lower gene expression (and less marked increment with
age) of MSA-A type II, than coeval WT animals. This altered expression pattern was already detectable at 2 mo of age and was maintained (and further enhanced) throughout the entire life span, thus confirming and extending our previous observations in diabetic and AGE-injected animals (23, 42).

These data demonstrate that age-related changes in renal function and structure are significantly correlated with the biochemical and molecular events associated with activation of the AGE/AGE receptor pathway, i.e., increased AGE levels, AGE receptor expression, ROS generation and oxidative stress, and NF-κB activity (56, 32, 6, 47). This correlation supports the concept of a central pathogenetic role for AGEs and AGE receptor-mediated events in the development of glomerular lesions in aged animals. More importantly, our study provides the novel finding that a more advanced glomerulopathy develops with aging in a model of increased susceptibility to AGE-induced tissue injury, such as the galectin-3 KO mouse (42, 23), in which the events associated with RAGE signaling

Fig. 4. Immunohistochemical analysis of fibronectin (A-D) and collagen IV (E-H) expression in kidney sections from WT and galectin-3 KO mice of 12 and 24 mo of age (original magnification ×1,000). A and E: WT-12 mo; B and F: KO-12 mo; C and G: WT-24 mo; D and H: KO-24 mo. Brown staining indicates glomerular positivity for fibronectin or collagen IV (arrows), which was significantly more pronounced in KO than WT mice, particularly at 24 mo of age.
and downregulation by galectin-3 are significantly more pronounced. In fact, galectin-3 seems to interfere with the redox-sensitive pathway triggered by RAGE, either directly or through the modulation of the expression of the other AGE receptors to favor AGE degradation vs. cell activation, thus providing protection toward AGE-dependent tissue injury (22).

Conversely, all of the concurrently assessed functional, structural, biochemical, and molecular parameters were similar in the young KO and WT mice. Taken together, our results provide strong experimental evidence linking AGEs, oxidative stress, and low-grade inflammation in the pathogenesis of age-related renal disease.

Aging and age-related disorders have been associated with chronic low-grade inflammation, although it is unclear whether the increased levels of proinflammatory cytokines and chemokines play a pathogenic role in the process of aging or reflect...
the presence of associated disorders (8). Aged female mice were found to develop progressive glomerular lesions associated with macrophage infiltration and a gene expression profile of intact glomeruli characterized by upregulation of inflammation-related genes, especially those expressed by activated macrophages, which might result from phenotypic proinflammatory changes in mesangial cells (59). The transcription factor NF-κB appears to play a major role in this scenario, since it participates in the control of cell-cycle, apoptosis, oxidative stress, immunity, inflammation, and repair, all functions that have been found to be profoundly altered during cellular senescence in cell culture systems and the aging process of humans and animals (14). The increased constitutive activity of NF-κB detected in tissues of aged animals (16, 17, 27, 30, 55) as well as in senescent cells (57) strongly supports this concept. In addition, most of the NF-κB target genes were shown to be modulated during senescence or aging by using transcriptome and proteome analysis (14). NF-κB has also been implicated in several renal disorders of an immune and nonimmune nature (15). It is activated by a variety of stimuli,
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ranging from cytokines to stress, particularly oxidative stress; there is great evidence that oxidizing conditions in the cytoplasma promote latent NF-κB dimers to be activated (34). AGE binding to RAGE is associated with generation of ROS, at least in part via stimulation of NADPH oxidase (53), and ROS-induced MAPK-dependent activation of NF-κB (32, 6) ultimately leading to tissue injury (47). Both CML (29) and HNE (26) have been shown to be capable of activating NF-κB. Thus this transcription factor seems to be the common final pathway of pathological conditions characterized by inflammation associated with altered cell and ECM turnover (15). These conditions include those in which AGES play a central pathogenetic role through the AGE/AGE receptor pathway, which is activated by RAGE and downregulated by galectin-3, such as diabetic glomerulopathy and, as indicated by our data, age-related renal disease. It is known that some beneficial effects of angiotensin-converting enzyme inhibitors and statins may, at least in part, be mediated by an inhibition of NF-κB activation (15). A better understanding of the mechanisms involved in NF-κB regulation by AGES and other pathological stimuli may contribute to the design of novel pharmacological interventions for treatment of several renal (and nonrenal) diseases.

In conclusion, the finding that age-dependent glomerular lesions were more pronounced in galectin-3 KO than WT mice supports the concept that the AGE/AGE receptor pathway (causing oxidative stress and chronic low-grade inflammation) is implicated in the pathogenesis of age-related renal disease and confirms the protective role of galectin-3 as an AGE receptor toward AGE-dependent tissue injury.

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