Abstract. Aging is associated with a progressive decline in renal function and the development of glomerulosclerosis and interstitial fibrosis. Although many studies have addressed the cellular mechanisms of age-related glomerulosclerosis, less is known about the tubulointerstitial fibrosis. In this study, aging (24 mo) rats develop tubulointerstitial fibrosis characterized by tubular injury and focal tubular cell proliferation, myofibroblast activation, macrophage infiltration with increased immunostaining for the adhesive proteins osteopontin and intercellular adhesion molecule-1, and collagen IV deposition. Aging rats demonstrated immunostaining for endothelial nitric oxide synthase (eNOSIII) in renal tubular epithelial cells and infiltrating mononuclear cells in areas of tubulointerstitial injury, with a relative loss of staining of the peritubular capillaries compared with young rats. The aging rats also displayed focal loss of peritubular capillaries (as noted by focally decreased RECA-1 and OX-2 staining) in areas of tubulointerstitial injury. The areas of fibrosis and hypocellularity were associated with increased apoptosis of tubular and interstitial cells compared with young (3 mo) rats (25.4 ± 5.3 versus 3.5 ± 2.5 TUNEL-positive cells/0.25 mm² in old versus young rats, P = 0.0001). It is concluded that tubulointerstitial fibrosis in aging is an active process associated with interstitial inflammation and fibroblast activation. The progressive loss of cells in areas of fibrosis may be due to accelerated apoptosis. Furthermore, the tubulointerstitial injury may be the consequence of ischemia secondary to peritubular capillary injury and altered eNOS expression. (J Am Soc Nephrol 9: 231-242, 1998)

Aging is associated with functional and structural changes in the kidney. The GFR of a term neonate is approximately 39 mL/min per 1.73 m², reaches adult levels by 2 yr of age (1), and is maintained at approximately 140 mL/min per 1.73 m² until 30 yr of age. At this point, the GFR declines in a roughly linear manner by approximately 8 mL/min per 1.73 m² per decade (2). Parallel reductions in renal blood flow occur (2-5) with redistribution of blood flow from the cortex to the medulla. Age-related changes in renal sodium conservation; urinary concentrating and diluting capacity; responsiveness to vasodilators; and renal functional reserve contribute to the decreased renal function with aging. As cortical renal blood flow declines, there is degeneration of cortical glomeruli with resultant glomerulosclerosis. In the juxtaglomerular region, shunts form between afferent and efferent arterioles, i.e., arteriolar everta (6) preferentially maintaining medullary blood flow. The classic structural finding with aging is glomerulosclerosis, leading to complete glomerular obsolescence and glomerular dropout. Several animal studies have confirmed this finding (7-9).

Much attention has been devoted to glomerulosclerosis with aging (2-4,7-12). However, few studies have been performed to characterize the tubulointerstitium (13), although reference to the tubulointerstitium is common. Nevertheless, numerous studies have shown that it is the degree of tubulointerstitial injury that best correlates with the degree of renal impairment and predicts the risk for progression (14-19). In addition, it has recently been hypothesized that it is the degree of tubulointerstitial injury that may be responsible for the impairment in sodium excretion in salt-dependent hypertension (20). Therefore, we decided to examine the role of the tubulointerstitium in age-related nephropathy in laboratory rats.

Materials and Methods
Experimental Design
Studies were conducted in old (24 mo) (n = 9; 568 ± 13 g) and young (3 mo) (n = 8; 377 ± 29 g) adult male Sprague Dawley rats (Harlan, Indianapolis, IN). To examine tubulointerstitial disease in age-related nephropathy, we performed two studies. In our first study, tissues from aging rats (n = 4) and young rats (n = 4) were examined by light microscopy and immunohistochemistry. Biopsies from a second set of aging rats (n = 5) and young rats (n = 4) with a histology comparable to the first group were studied for apoptosis by periodic acid-Schiff (PAS), propidium iodide, and terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin nick end labeling.
(TUNEL). All rats were fed standard rat chow (Rodent Laboratory Chow 5001, Ralston Purina, Richmond IN) ad libitum. They were housed in an animal facility with standard 12-h light/dark cycles. Systolic BP was measured by the awake tail-cuff method (21). For determination of proteinuria, animals were placed in individual metabolic cages for 24-h urine collections. Urine protein was measured by precipitation with 3% sulfosalicylic acid. To obtain histologic samples, rats were anesthetized with inactin (100 mg/kg, intraperitoneally), and the kidneys were quickly harvested and placed in either 10% buffered formalin phosphate or methyl Carnoy’s solution, followed by immersion in 70% methanol after 24 h (22). These studies were approved by the Portland Veterans Administration Institutional Animal Care and Use Subcommittee.

Renal Morphology and Immunohistochemistry

Methyl Carnoy’s fixed tissue was paraffin-embedded and processed, and 4-μm sections were stained with PAS reagent, Masson’s trichrome, and silver stain. Additional 4-μm sections were immunostained using an indirect avidin-biotin immunoperoxidase method with specific monoclonal and polyclonal antibodies directed to the following antigens (22): the proliferating cell nuclear antigen (PCNA)/cyclin with 19A2 (Coulter, Miami FL); alpha-smooth muscle actin with 1A4 (Sigma Immunochemicals, St. Louis, MO); type IV collagen with goat antihuman and bovine anticollagen IV (Southern Biotechnology, Birmingham, AL); endothelial nitric oxide synthase with anti-ECNOS clone 3 (Transduction Laboratories, Lexington, KY); endothelial cells with monoclonal rat antienothelial cell antibody RECA-1 (kind gift of A. Duijvestijn, Maastricht, The Netherlands); membrane glycophosphatidylinositol, neuronal, endothelial, and follicular dendritic cells with monoclonal mouse antibody OX-2 (Serotec, Indianapolis, IN); monocyte-macrophages with mouse monoclonal ED-1 (Serotec); common leukocyte antigen present on all leukocytes, but predominantly expressed by macrophages; T and B lymphocytes with monoclonal OX-1 (Accurate Chemical and Scientific Corp., Westbury, NY); osteopontin with goat antiosteopontin antibody OPUS (kind gift of C. Giachelli, University of Washington, Seattle, WA); and intercellular adhesion molecule-1 (ICAM-1) with clone 1A29 (Caltag Laboratories, Burlingame, CA).

Apoptotic cells were detected in tissue sections from young and aging animals by the TUNEL assay as described previously (23). Briefly, 4-μm formalin-fixed sections were deparaffinized and rehydrated in ethanol, followed by an antigen retrieval step using boiling citric acid (pH 6.0; 10 mM). Samples were incubated in proteinase K (6.2 μg/ml; Boehringer Mannheim, Indianapolis, IN), followed by Tdt (300 enzyme units/ml; Pharmacia Biotech, Alameda, CA) and Bio-14-dATP (0.94 nm; Life Technologies, Grand Island, NY). Biotinylated ATP was detected using the Vectastain Elite ABC peroxidase staining kit (Vector Laboratories, Burlingame CA) following the manufacturer’s protocol. As a positive control, slides were treated with DNase (20 Kunitz units/ml; Sigma Biosciences, St. Louis, MO). The slides for the negative control were treated with buffer lacking Tdt. Apoptosis was also documented by propidium iodide staining as described previously (24).

Quantification of Morphological Data

The mean number of proliferating cells (PCNA-positive), macrophages (ED-1-positive cells), and TUNEL-positive cells in each biopsy was calculated in a blinded manner by counting the total number of positive cells in 30 sequentially selected 0.25 mm² grids at 200× magnification and summed. Interstitial fibrosis was scored semiquantitatively on biopsies stained with PAS, Masson’s trichrome, and silver stain, using the following scoring system: 0 = normal interstitium and tubules; 1 = mild fibrosis with minimal thickening between the tubules; 2 = moderate fibrosis with moderate interstitial thickening between the tubules; 3 = severe fibrosis with severe interstitial thickening between the tubules. A 0 to 3 scoring system was also used for interstitial alpha-smooth muscle actin, interstitial collagen IV, OX-1-positive cells, osteopontin, and interstitial ICAM-1. (0 = normal interstitium and tubules; 1 = mild; 2 = moderate; 3 = severe).

To quantify the degree of glomerulosclerosis in each biopsy, 50 sequential glomeruli were counted per biopsy in a blinded manner, and the following scoring system was developed for each glomerulus: 0 = 0% glomerulosclerosis; 1 = 25% glomerulosclerosis (one-quarter of the glomerulus); 2 = 50% glomerulosclerosis, (one-half of the glomerulus); 3 = 75% glomerulosclerosis (three-quarters of the glomerulus); and 4 = 100% glomerulosclerosis (global glomerulosclerosis).

Statistical Analysis

All values are expressed as mean ± SD, unless otherwise stated. Statistical significance (P < 0.05) was evaluated using the unpaired t test.

Results

Glomerular Changes

Values for systemic and renal physiologic parameters in the young and old rats are depicted in Table 1. Values for body weight and right kidney weight were higher in old rats, but the ratio of kidney/body weight with age was not statistically significant. Systolic BP was comparable in the young and old rats, indicating absence of hypertension with aging in this strain provided standard rat chow. Values for proteinuria were markedly increased in the older rats, indicating substantial renal injury.

Glomeruli in young and old rats were examined by PAS, silver stain, and Masson’s trichrome stain. The glomeruli in two of the four young rats were normal. One young rat exhibited mild mesangial matrix expansion in one of 50 glomeruli. The fourth young rat had 50% and global sclerosis in two glomeruli, respectively; the remaining 48 glomeruli examined had normal histology (Table 2) (Figure 1).

Glomeruli in the 24-mo-old rats exhibited various degrees of glomerulosclerosis, as has been frequently reported in the literature (Table 2). Glomeruli displaying focal segmental and global glomerulosclerosis were often associated with areas of tubulointerstitial involvement. Capillary loops within sclerotic

<table>
<thead>
<tr>
<th>Parameter parametersa</th>
<th>Young (n = 8)</th>
<th>Old (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>377 ± 29</td>
<td>568 ± 13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RKW (g)</td>
<td>1.35 ± 0.10</td>
<td>2.23 ± 0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RKW/100 g BW</td>
<td>0.36 ± 0.01</td>
<td>0.40 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>121 ± 3</td>
<td>131 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>Urine protein (mg/24 h)</td>
<td>9 ± 2</td>
<td>98 ± 21</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a BW, body weight; RKW, right kidney weight; NS, not significant.
**Table 2.** Changes in glomerular sclerosis score with aging

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Age (mo)</th>
<th>Sclerosis Score</th>
</tr>
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<tbody>
<tr>
<td>12531</td>
<td>3</td>
<td>100% = 0</td>
</tr>
<tr>
<td>12532</td>
<td>3</td>
<td>96% = 0, 2% = 2, 2% = 4</td>
</tr>
<tr>
<td>12533</td>
<td>3</td>
<td>100% = 0</td>
</tr>
<tr>
<td>12534</td>
<td>3</td>
<td>100% = 0</td>
</tr>
<tr>
<td>12535</td>
<td>24</td>
<td>56% = 0, 32% = 1, 10% = 2, 2% = 4</td>
</tr>
<tr>
<td>12536</td>
<td>24</td>
<td>38% = 0, 14% = 1, 14% = 2, 8% = 3, 26% = 4</td>
</tr>
<tr>
<td>12537</td>
<td>24</td>
<td>50% = 0, 32% = 1, 12% = 2, 4% = 3, 2% = 4</td>
</tr>
<tr>
<td>12538</td>
<td>24</td>
<td>34% = 0, 18% = 1, 14% = 2, 16% = 3, 18% = 4</td>
</tr>
</tbody>
</table>

**Figure 1.** Glomerular changes associated with aging. (a) Photomicrographs show, in young rats, normal glomerular architecture by periodic-acid Schiff (PAS). In contrast, 24-mo-old rats display varying degrees of glomerulosclerosis. A glomerulus with focal segmental glomerulosclerosis (PAS; Panel b) and global glomerulosclerosis (PAS; Panel c). (d) Occasional glomeruli also displayed synechiae formation (silver stain; arrow). Magnification, ×400.

lobules were obliterated, whereas those at the periphery remained patent. Sclerotic lobules often formed synechiae with Bowman’s capsule. Mesangial matrix expansion and thickening of the glomerular capillary basement membranes were also visualized (Figure 1).

### Tubulointerstitial Changes

**General Histology.** Examination of the tubulointerstitium of 3-mo-old rats by PAS, silver stain, and Masson’s trichrome stain revealed normal histology. Rats at 3 and 24 mo of age had normal-appearing vasculature by routine light microscopy, with no evidence of endothelial injury, medial hypertrophy, or perivascular fibrosis. However, the rats at 24 mo of age had pronounced tubulointerstitial injury, with large areas displaying tubular dilation, intratubular cast formation, tubular atrophy, thickening and splitting of tubular basement membranes, and widening of the interstitium with fibrosis (Table 3) (Figure 2). Notably, an inflammatory infiltrate consisting of mononuclear cells was found to be colocalized with areas of tubulointerstitial injury.

**Tubular Proliferation/Apoptosis.** Immunostaining with 19A2, a murine IgM monoclonal antibody against human PCNA that is expressed by actively proliferating cells, was performed on tissues from young and aging rats. The mean number of PCNA-positive nuclei per 30 fields in the young rats was 44.2 ± 15.0 compared with 55.0 ± 18.1 in the aging rats.
Table 3. Summary of tubulointerstitial changes in aging rats

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Age (mo)</th>
<th>Fibrosis</th>
<th>PCNA</th>
<th>Actin</th>
<th>Coll IV</th>
<th>OPN</th>
<th>ICAM-1</th>
<th>ED-1</th>
<th>OX-1</th>
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<td>59</td>
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<tr>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>58</td>
<td>0</td>
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<tr>
<td>12533</td>
<td>3</td>
<td>35</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>68</td>
<td>0</td>
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</tr>
<tr>
<td>12534</td>
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<td>28</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>33</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12535</td>
<td>24</td>
<td>71</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>157</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>12536</td>
<td>24</td>
<td>70</td>
<td>3</td>
<td>3</td>
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<td>3</td>
<td>247</td>
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<tr>
<td>12537</td>
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<td>43</td>
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<td>2</td>
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<td>2</td>
<td>92</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>12538</td>
<td>24</td>
<td>36</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>153</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* PCNA- and ED-1-positive cells/30 (0.25 mm²) fields at ×200 magnification. PCNA-positive cells young (44.2 ± 15.0) versus old (55.0 ± 18.1) (P = 0.39). ED-1-positive cells young (47.2 ± 18.6) versus old (162.2 ± 3.8) (P = 0.01). PCNA, proliferating cell nuclear antigen; Coll IV, collagen IV; OPN, osteopontin; ICAM-1, intercellular adhesion molecule-1; ED-1, monocyte/macrophage; OX-1, common leukocyte antigen.

Figure 2. Tubulointerstitial changes associated with aging. Photomicrographs show, in young rats, normal tubules and interstitium by PAS (a) and silver stain (b). Aging rats display significant tubulointerstitial injury evidenced by tubular dilation, tubular atrophy, intratubular cast formation, and extracellular matrix expansion by PAS (c) and by silver stain (d). (d) Basement membrane splitting in 24-mo-old rats (arrow). Magnification: ×200 in c; ×400 in a, b, and d.

Although the difference in proliferation was not statistically significant, PCNA-positive nuclei were dispersed between the glomeruli, tubules, and interstitium in the young rats, whereas in the aging rats PCNA-positive nuclei were concentrated within tubules and glomeruli in areas of injury, and in the surrounding mononuclear infiltrates (Figure 3).

To determine the role that apoptosis plays in the development of the hypocellular lesions seen in tubulointerstitial fibrosis, we quantified TUNEL-positive cells in the biopsies of young and old rats. Although there was not a statistically significant difference in proliferation, there was a marked difference in apoptosis seen in the aging rats (young 3.5 ± 2.5 versus old 25.4 ± 5.3; P = 0.0001) (Table 4). Both tubular and interstitial cells showed increased apoptotic rates. The presence of apoptosis was confirmed by PAS and propidium iodide staining (Figure 3).

Phenotype Change: Myofibroblast Activation/Matrix Expansion. To study the composition of the expanded renal interstitium in aging rats, biopsies were stained for alpha-smooth muscle actin and collagen IV. Alpha-smooth muscle...
Figure 3. Cellular proliferation and apoptosis in normal and aging rats. Photomicrographs show, in young rats, proliferating cell nuclear antigen (PCNA)-positive cells in the tubules, glomeruli, and interstitium (a, arrows), and in aging rats PCNA-positive tubular epithelial cells and infiltrating mononuclear cells localized to areas of injury (b, arrows). A notable increase in the number of apoptotic cells in aging rats was detected by terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) (young rats, panel c, and aging rats, panel d). Confirmation of apoptosis was performed by PAS (e and f, arrows) and by propidium iodide staining (g). Magnification: ×400 in a through d; ×1000 in e and f; ×630 in g.
Table 4. Summary of apoptosis in aging rats*  

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Age (mo)</th>
<th>TUNEL+ cells/30 fields</th>
<th>Glomerular</th>
<th>Tubular</th>
<th>Interstitial</th>
</tr>
</thead>
<tbody>
<tr>
<td>12875</td>
<td>3</td>
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</tr>
<tr>
<td>12876</td>
<td>3</td>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>12877</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>12878</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.5 ± 2.5</td>
<td>1.0 ± 1.2</td>
<td>2.3 ± 2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* TUNEL-positive cells/30 (0.25mm²) fields at ×200 magnification. TUNEL, terminal deoxynucleotidytransferase-mediated dUTP biotin nick end labeling.  

- P = 0.0001.  
- P = 0.003.  
- P = 0.0004.

actin is normally expressed by smooth muscle cells, myoepithelial cells, and vascular pericytes. Interstitial cells and mesangial cells with the characteristics of myofibroblasts have also been shown to express alpha-smooth muscle actin (21,25). These myofibroblasts have been shown to participate in chronic tubulointerstitial injury. Biopsies from young rats showed typical alpha-smooth muscle actin staining of the afferent and efferent arterioles, intrarenal arteries, and the vasa rectae. Tissues from old rats showed a vascular staining pattern similar to the young rats, with the additional staining of mesangial cells, and interstitial fibroblasts surrounding dilated and atrophic tubules and Bowman’s capsule (Table 3) (Figure 4). In addition to the above change, there was marked type IV collagen staining in the interstitium (produced either by injured tubules or interstitial fibroblasts), and in the sclerotic glomeruli of aging rats (Table 3) (Figure 4).

**Peritubular Capillaries**

To investigate potential vascular changes in aging rats, tissues were immunostained for endothelial nitric oxide synthase (eNOS). Both young and old rats exhibited eNOS staining of vascular endothelium, visceral and parietal epithelial cells, and glomerular endothelial cells (Figure 5). However, the most intriguing finding was the differential eNOS immunostaining in the young versus the old rats. Aging rats demonstrated focal increases in eNOS staining of tubules and infiltrating mononuclear cells in areas of tubulointerstitial injury, whereas there was a generalized reduction of eNOS staining in the peritubular capillaries throughout the cortex (Figure 5). In contrast, the young rats displayed a greater degree of eNOS staining of the peritubular capillaries, with a relative lack of tubular staining (Figure 5). Staining of the peritubular capillaries in the four young rats was segmental in distribution, and more pronounced in two of the four young rats. Nevertheless, the degree of eNOS staining in the peritubular capillaries was more pronounced in the young rats, and the differential immunostaining appeared to be significant.

To determine whether the differential eNOS staining of the peritubular capillaries was associated with alterations in peritubular capillary structure, tissues were stained with the monoclonal rat antiendothelial cell antibody RECA-1. Both animal groups demonstrated staining of vascular endothelial cells and glomerular endothelial cells. In areas characterized primarily by tubular dilation, as well as in more injured areas with tubular atrophy and fibrosis, focal decreases in peritubular capillary staining were observed (Figure 5). To confirm this finding, endothelial cell staining was performed with a second monoclonal antibody to vascular endothelium (OX-2). OX-2 is a monoclonal antibody that stains vascular endothelium, as well as glycoproteins of thymocytes, neuronal and follicular dendritic cells, and has minimal reactivity with vascular smooth muscle cells. Although OX-2 is not as specific for the endothelium as RECA-1, immunostaining with OX-2 shows a similar pattern, with loss of peritubular capillary staining in areas of tubular damage and fibrosis (Figure 5).

**Inflammatory Infiltrates**

The tubular injury in the aging rats was associated with a generalized as well as focal macrophage infiltration in the interstitium, as determined by immunostaining with the specific monoclonal antibody ED-1 (162.2 ± 63.8 versus 47.2 ± 18.6 cells/mm², P = 0.01). There also was a substantial increase in the number of leukocytes detected by the OX-1 antibody (primarily lymphocytes and macrophages) in the interstitium of the aging rats versus the young rats. Large concentrations of inflammatory cells were seen in association with tubular dilation, tubular atrophy, and interstitial fibrosis (Table 3) (Figure 6).
Adhesion Molecules
Osteopontin, also known as uropontin, is a highly acidic, secreted glycoprotein found in many tissues, including the kidney. It has been shown that osteopontin expression is increased in various models of tubulointerstitial injury, and the colocalization of osteopontin-positive tubules and ED-1-positive inflammatory cells suggests that one of the roles of osteopontin is to facilitate monocyte/macrophage accumulation at sites of renal damage (26). Consistent with these findings, tubulointerstitial injury in aging rats was associated with a marked increase in cortical proximal and distal tubular osteopontin immunostaining compared with young rats (Table 3) (Figure 7).

Immunohistochemistry for ICAM-1 revealed increased staining of the interstitium surrounding areas of tubular injury, frequently associated with positive-staining mononuclear cell infiltrates. In addition, there was staining of the brush borders of tubular epithelial cells, the peritubular capillaries in regions of tubulointerstitial disease, and glomerular endothelial cells in diseased glomeruli. Evaluation of tissue from young rats revealed only faint staining of occasional peritubular capillaries (Table 3) (Figure 7).

Discussion
Although age-related glomerulosclerosis has been reported frequently in the literature (2–4,7–12), fewer studies have been
Figure 5. Peritubular capillary and endothelial nitric oxide synthase (eNOS) immunostaining are altered in the aging rat. Photomicrographs show differential eNOS immunostaining and peritubular capillary structure in young and aging rats. (a) Immunostaining for eNOS by the peritubular capillaries in the young rats. Note the relative absence of eNOS staining in the peritubular capillaries of the aging rats (b). (c) eNOS immunostaining by tubular epithelial cells in areas of tubulointerstitial injury (large arrows), as well as by infiltrating cells (small arrows). (d) eNOS staining in some glomeruli of young and aging rats. This photomicrograph was taken from an aging rat, but similar findings were found in various young rat glomeruli. Note the eNOS staining by the visceral epithelial cells (thick black arrows) and glomerular endothelial cells (thin black arrow). Parietal epithelial cells were also positive in some glomeruli (not shown). Normal peritubular capillary architecture by RECA-1 staining in the young rat (e). In contrast, note the areas of patchy loss of peritubular capillary staining by RECA-1 in the aging rat in areas of tubulointerstitial injury (f). Confirmation of these findings was performed by staining for the peritubular capillaries with OX-2. Normal peritubular architecture in the young rat (g, arrows) compared with the lack of staining in areas of tubular dilation in the aging rat (h). Magnification: ×400 in a through c, g and h; ×630 in d through f.
Figure 6. Age-associated tubulointerstitial disease is associated with a prominent inflammatory infiltrate. Photomicrographs show a prominent inflammatory infiltrate in the tubulointerstitium of aging rats. Occasional ED-1-positive monocyte/macrophages (a) and OX-1-positive monocyte/macrophages (b) and OX-1-positive lymphocytes/macrophages (d) in areas of tubulointerstitial injury; and differential immunostaining for eNOS in young and aging rats. Magnification, ×400.

performed to describe age-related tubulointerstitial changes (13). The purpose of this study was to identify the cellular processes contributing to the development of tubulointerstitial fibrosis. As a result, several interesting findings were observed, including a prominent inflammatory infiltrate; increased immunostaining for the leukocyte adhesion molecules osteopontin and ICAM-1; myofibroblast activation; focal proliferation of tubular epithelial cells and infiltrating mononuclear cells without an overall increase in proliferation; a marked increase in apoptosis of tubular epithelial cells and interstitial cells (presumably interstitial fibroblasts and infiltrating macrophages); focal peritubular capillary loss at sites of tubulointerstitial injury; and differential immunostaining for eNOS in young and aging rats.

The first major finding was the observation that a prominent leukocyte infiltration occurs. These cells are composed of monocyte/macrophages and lymphocytes. This was associated with increased immunostaining for the leukocyte adhesion molecules osteopontin and ICAM-1. There have been few reports of osteopontin and its role in the aging process. Liang and Barnes showed that osteopontin mRNA levels were elevated in old rats by Northern blot hybridization and that renal expression of osteopontin correlated with blood urea nitrogen in aged rats (27). An additional report by Lopez et al. studied osteopontin via immunohistochemistry and in situ hybridization and found that as mice aged, expression of osteopontin was found in more proximal portions of the tubule (28). They also found that osteopontin expression was markedly increased in the parietal epithelium of glomeruli undergoing sclerosis in aging mice. On the other hand, osteopontin has been repeatedly associated with tubulointerstitial disease in such models as exogenous angiotensin II infusion (26), cyclosporine (17), mesangial proliferative nephritis (the Thy 1 model) (29), focal glomerulosclerosis (the aminonucleoside or puromycin aminonucleoside nephrosis) (29), and membranous nephropathy (passive Heymann nephritis) (29). Similar to the findings of Nikolic-Paterson et al. (30), in which ICAM-1 immunostaining was found to be increased in the tubulointerstitium in a model of glomerulonephritis, we have also shown an increase in ICAM-1 immunostaining within the tubulointerstitium that was associated with leukocyte infiltration and tissue injury. These interstitial cells likely represent leukocytes and interstitial fibroblasts. Nikolic-Paterson et al. have also shown that interleukin-1 is a major inducer of ICAM-1 expression within the renal tubulointerstitium by the administration of the interleukin-1 receptor antagonist in their model (30).

A second major finding that has been previously associated with tubulointerstitial injury and is now shown to be involved in age-related nephropathy is the phenotypic modulation of mesangial cells and interstitial fibroblasts. As a result, these cells express alpha-smooth muscle actin, a protein usually associated with smooth muscle cells. These myofibroblasts are
involved in wound healing and wound contracture, and have been shown to participate in other forms of tubulointerstitial disease such as angiotensin II (Ang II)-mediated renal injury \((21)\). It appears that the renal interstitial fibroblast and mesangial cell become activated in aging and Ang II-mediated injury, assume characteristics of "myofibroblasts," proliferate, express alpha-smooth muscle actin, and secrete collagens \((21)\). It is also possible that tubular epithelial cells contribute to collagen IV production.

The third major finding was that an imbalance occurred between proliferation and apoptosis. Although there was an increase in proliferation of tubular epithelial cells and infiltrating mononuclear cells in areas of injury, the difference in proliferation seen between the young and aging rats was not statistically significant. This is in contrast to other models of tubulointerstitial injury, including the remnant kidney model \((15)\), in which proliferation was a prominent finding. Interestingly, apoptosis of tubular epithelial cells and interstitial cells (presumably interstitial fibroblasts and infiltrating leukocytes) increased in age-related nephropathy. Therefore, it appears that increased apoptosis may contribute to the progressive hypocellularity associated with age-related nephropathy.

One of the most exciting findings was evidence that the peritubular capillaries were injured. Although the vasculature by PAS and alpha-smooth actin staining appeared normal, visualization of the peritubular capillaries is difficult by routine light microscopy. Using two different antibodies (RECA-1 and OX-2) that stain endothelial cells, we found loss of staining for endothelial cells in areas of tubulointerstitial injury, suggesting a loss of the peritubular capillaries themselves. This rarefaction of peritubular capillaries may be contributing to the development of fibrosis in these areas, since these findings were colocalized. In support of this, Bohle et al. has postulated that human tubulointerstitial injury is associated with a decrease in the number and area of postglomerular capillaries as interstitial fibrosis of the renal cortex interstitium increases and as serum creatinine rises \((14,16,31,32)\). This leads to an increase in resistance in the postglomerular region, as well as local ischemia to the surrounding tubules and interstitium, and could account for the tubulointerstitial changes observed.

Another exciting finding was the observation of a relative loss of immunostaining for eNOS in the peritubular capillaries of the aging rats that may indicate a decrease in the level of this enzyme. This included areas of normal-appearing cortex, suggesting that this is a primary event. eNOS plays a role in mediating vasodilation, inhibition of platelet activation, and in interfering with leukocyte adhesion by inhibiting the synthesis of monocyte chemoattractant protein-1 at the transcriptional level \((33)\). Thus, downregulation of eNOS could contribute to renal ischemia. Alterations in endothelial nitric oxide synthesis

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**Figure 7.** Age-associated tubulointerstitial disease is associated with an increase in immunostaining of leukocyte adhesion proteins. Photomicrographs show increased immunostaining for the leukocyte adhesion proteins osteopontin and ICAM-1 in aging rats. Young rats display very little osteopontin in renal tubules (a, arrow) compared with the marked staining in proximal and distal tubules in aging rats (b, arrow). ICAM-1 was noted in occasional peritubular capillaries in biopsies from young rats (c, arrow). In comparison, note the increased immunostaining of interstitial ICAM-1 surrounding areas of tubular injury (d, thick black arrow), as well as the characteristic staining of the tubular epithelial brush borders (d, thin black arrow). Magnification, \(\times 400\).
or release have been associated with aging (34). Campo et al. compared the renal response to an intravenous infusion of L-arginine in young and aged essential hypertensive patients (34). In young hypertensive patients, L-arginine induced a significant increase in renal plasma flow, GFR, natriuresis, and kaliuresis, without changes in filtration fraction. These effects were not observed in the aged hypertensive group (34). Interestingly, immunostaining for eNOS was observed in tubular epithelial cells and infiltrating mononuclear cells in areas of tubulointerstitial injury. Although eNOS is typically considered endothelial and constitutive, neither is absolute. Indeed, eNOS has been identified in activated fibroblasts (35), red blood cells (36), monocytes/macrophages (37), T and B lymphocytes (38), and polymorphonuclear leukocytes (39). It is possible that eNOS production by infiltrating leukocytes and tubular epithelial cells is a compensatory mechanism for the loss of eNOS expressed in the peritubular capillaries.

The mechanism for the decrease in eNOS is unclear. It could relate to the rise in intrarenal Ang II associated with aging (40). It has been shown that angiotensin-converting enzyme inhibition slows the development of both glomerular and tubulointerstitial injury in aging rats (10). Little is known about the mechanisms of Ang II-induced injury in the aging kidney. Of note, Ang II increases renal osteopontin (26), platelet-derived growth factor (21), type IV collagen (21), and TGF-β1 (41) in other models, all of which may be important mediators of tubulointerstitial disease. The present findings of substantial tubulointerstitial fibrosis, together with the observation of elevated intrarenal Ang II levels, presumably in tubulointerstitial locations (40), suggest that the renin-angiotensin system may be an important mediator of fibrosis in the aging kidney. Studies in models of vascular injury have identified an inverse relationship with local Ang II and eNOS, in which elevated Ang II is associated with a reduction in eNOS (42). Specific blockade of Ang II with an AT1 receptor antagonist normalizes eNOS availability, suggesting a cause-and-effect relationship. Another potential mechanism is vascular injury (i.e., peritubular capillaries, glomeruli) leading to hypoxia, because hypoxia has been reported to inhibit eNOS production via transcriptional and posttranscriptional mechanisms (43).

In conclusion, tubulointerstitial fibrosis is an active process associated with an inflammatory infiltrate, increased immunostaining for leukocyte adhesion molecules, myofibroblast activation, and type IV collagen deposition. The ultimate result is tubular injury, with an imbalance between proliferation and apoptosis leading to hypocellularity. The mechanism for the relative decrease in aging peritubular capillary eNOS expression is unknown. However, focal loss of peritubular capillaries and differential eNOS immunostaining as shown in this study provide insight into alterations that occur in the renal microvasculature as a result of aging. These findings may contribute to the development of tubulointerstitial ischemia and tubulointerstitial injury associated with aging.

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