Glomerular Metalloprotease Activity in the Aging Rat Kidney: Inverse Correlation With Injury

Jane F. Reckelhoff and Christine Baylis

Glomerular metalloprotease activity (phenanthroline inhibitable) was measured in cortical homogenates from rat kidneys by use of the rate of degradation of $^3$H-denatured type I collagen (gelatin). Glomerular metalloprotease activity was similar in male and female kidneys from young (4 months) rats ($27 \pm 7$ and $34 \pm 6 \mu$g of $^3$H-gelatin degraded/h per milligram of glomerular protein, respectively). Glomerular metalloprotease activity was unaltered by age (18 to 20 months) in intact males but increased two times in old intact females, castrated males, and ovariectomized females. These aging changes correlated inversely with the level of age-dependent glomerular injury, i.e., old intact males had substantially greater glomerular damage than all other old groups. These observations suggest (1) that the androgens are a risk factor for the development of age-dependent glomerular damage; and (2) that an age-dependent increase in glomerular metalloprotease activity may protect against damage by limiting the build-up of glomerular extracellular matrix.

Aging in the renal glomerulus is characterized by thickening of the glomerular basement membrane, increased mesangial matrix content (predominantly collagen), and the development of glomerular sclerotic injury; glomerular function also declines with age (1-3). In several glomerular diseases, damage is preceded by proliferation of mesangial cells and accumulation of glomerular extracellular matrix material, events thought to play a primary pathogenic role in glomerular injury (3,4). A threefold increase in the hydroxyproline content of rat glomeruli (a marker for collagens) occurs when rats age from 5 wk to 2 yr, and even when the increase in glomerular volume is accounted for, there is still a twofold increase in glomerular hydroxyproline content over this period (5). This accumulation of glomerular extracellular matrix may be a result of either increased matrix production and/or decreased matrix degradation. The glomerulus produces a number of metalloproteases, and it is possible that they may play a role in glomerular injury (4,6-8). These studies were conducted to measure glomerular metalloprotease activity in glomerular homogenates obtained from old and young rats. Because females are markedly protected against the age-dependent glomerular injury seen in males (2), we also conducted experiments in aging intact females and in aging castrated rats of both sexes and compared them with young intact males and females.

METHODS

Male and female Munich Wistar rats, aged either 4 months or 18 to 20 months, were housed from birth on a 12-h-light/12-h-dark cycle in a barrier-maintained facility (Simonsen, Gilroy, CA) to eliminate airborne disease, with ad libitum access to standard laboratory chow (approximately 20% protein, 1% NaCl) and tap water. For some of the studies, male rats were castrated and females were ovariectomized at 10 wk of age and allowed to age to 18 to 20 months. Two to four weeks before the study, rats were shipped to our laboratory. On the day of study, rats were anesthetized with the thiobarbiturate methohexitol (120 mg/kg body wt, ip; Byk Gulden, Konstanz, Germany), and one kidney was removed, weighed, hemisected, and placed in 10% buffered formaldehyde (see below) whereas the other kidney was perfused bloodless in situ with phosphate-buffered saline (PBS). The glomeruli were isolated by differential sieving in PBS by the method of Foldart et al. (9) and were collected by centrifugation (600 rpm; 4 min; 4°C). This technique yielded a >90% pure preparation.
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of cortical glomeruli. After centrifugation, the PBS was aspirated and 1 mL of 50 mM Tris (pH 7.6) with 200 mM NaCl was added to the glomeruli. Glomeruli were kept on ice and homogenized by sonication (3 x 1 min) with 1-min cool-down periods between bursts. Samples of glomerular homogenate were stored at −20°C in 100-μL aliquots until assayed. Protein concentrations of glomerular samples were determined by the method of Lowry et al. (10).

Denatured type I collagen (gelatin) (Sigma Chemical Company, St. Louis, MO), with 2H]acetic anhydride (NEN Research Products, Wilmington, DE) Glomerular metalloprotease activity was measured with tritiated gelatin as substrate by the method of Martin et al. (8). Briefly, 25 μL of glomerular homogenate (approximately 25 μg) was incubated in microfuge tubes in shaking water bath with 100 μg of [3H]gelatin (=10,000 cpm) in a final concentration of 160 mM Tris (pH 7.6)-80 mM CaCl2-0.01% Brij-35 for 18 h at 37°C. The reaction was stopped with the addition of 50 μL of ice cold 2% BSA and 300 μL of 25% trichloroacetic acid. After centrifugation, radioactivity in an aliquot of the supernatant was counted by liquid scintillation. The glomerular metalloprotease activity was expressed as percent inhibition of [3H]gelatinase activity. Glomerular metalloprotease activity was measured in duplicate at least four times per sample, and phenanthroline inhibition was measured in duplicate in at least three assays for each sample.

The kidney, fixed in 10% formaldehyde, was later dehydrated in ethanol and blocked in paraffin wax and 3-μm-thick sections were cut and stained with periodic acid-Schiff with hematoxylin/eosin counterstain. Glomerular sclerosis was evaluated, blinded, on 100 cortical glomeruli/section, with each section evaluated twice. The glomeruli evaluated histologically were from the same (cortical) population as those isolated by differential sieving for the measurement of glomerular metalloprotease activity.

RESULTS

Body weights were higher in males compared with females, both young and old (Table 1; Groups 1, 3, and 5 versus Groups 2 and 4); however, aged ovariectomized females were heavier than intact females and had body weights similar to those of young males. Kidney weights were also higher in intact males versus females, although this difference disappeared when factored for body weight. Castration had no effect on kidney growth with advancing age in males or females, but because ovariectomy increased body weight, the kidney weight/body weight ratio was re-

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Kidney wt (g)</th>
<th>Kidney wt/Body wt (%)</th>
<th>MP Activity (μg/h·mg)</th>
<th>Inhibition of Phenanthroline (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, young males (N = 5)</td>
<td>286 ± 16</td>
<td>1.44 ± 0.08</td>
<td>0.504 ± 0.004</td>
<td>27.0 ± 7.0</td>
<td>98.4 ± 1.3</td>
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<td>2, young females (N = 5)</td>
<td>172 ± 4</td>
<td>0.85 ± 0.05</td>
<td>0.491 ± 0.020</td>
<td>34.5 ± 6.3</td>
<td>97.5 ± 0.9</td>
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<td>3, aged males (N = 7)</td>
<td>399 ± 6</td>
<td>1.55 ± 0.04</td>
<td>0.390 ± 0.150</td>
<td>31.3 ± 5.4</td>
<td>99.7 ± 0.2</td>
</tr>
<tr>
<td>4, aged females (N = 5)</td>
<td>238 ± 6</td>
<td>1.15 ± 0.04</td>
<td>0.482 ± 0.020</td>
<td>64.4 ± 5.4</td>
<td>99.2 ± 0.5</td>
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<tr>
<td>5, aged, castrated males (N = 3)</td>
<td>393 ± 2</td>
<td>1.37 ± 0.05</td>
<td>0.350 ± 0.020</td>
<td>65.3 ± 3.8</td>
<td>96.3 ± 1.4</td>
</tr>
<tr>
<td>6, aged, ovariectomized females (N = 4)</td>
<td>304 ± 8</td>
<td>1.11 ± 0.09</td>
<td>0.363 ± 0.030</td>
<td>67.9 ± 7.9</td>
<td>95.5 ± 1.9</td>
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</tbody>
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Table 1. Glomerular metalloprotease activity in intact young (4 months) and old (18 to 20 months) male and female Munich Wistar rats and in old rats (18 to 20 months castrated or ovariectomized at 10 wk of age). Glomerular metalloprotease activity was measured in duplicate at least four times per rat and is expressed as micrograms of [3H]gelatin degraded per hour per milligram of protein. Statistical analysis by analysis of variance. NS, not significant.
duced. Glomerular metalloprotease activity was similar in young intact males and females and in old males (Groups 1, 2, and 3). However, glomerular metalloprotease activity in old females (Group 4) was approximately twofold higher than the activity in either young or old males (Groups 1 and 3) or young females. Elevated glomerular metalloprotease activity was also seen in old castrated males (Group 5) and old ovariectomized females (Group 6). In the presence of the metalloprotease inhibitor phenanthroline, proteolytic activity was inhibited by >95% in all groups, indicating that the substrate gelatin (collagen) was exclusively degraded by glomerular metalloproteases.

The level of glomerular sclerotic injury was evaluated, blinded, on a 0 to 4+ scale for all four groups of old rats. The old intact males had significantly fewer normal glomeruli (83 ± 2%) compared with the intact females, ovariectomized females, and castrated males (91 ± 2, 92 ± 1, and 92 ± 2%, respectively; all P < 0.05 versus intact males). The level of focal sclerosis (1+ and 2+ injury, denoting ~25 and ~50% glomerular damage, respectively) was similar between old intact males (7 ± 1 and 2 ± 1%) and old castrated males (4 ± 1 and 2 ± 1%), old intact females (7 ± 1 and 2 ± 1%), and old ovariectomized females (5 ± 1 and 2 ± 1%). However, the old intact males had more severe glomerular sclerosis (3+ to 4+, denoting >75% injury and global sclerosis, respectively; 3 ± 1 and 4 ± 1%; both P < 0.05) compared with old castrated males (0 ± 0 and 1 ± 0%), old intact females (1 ± 0% and 1 ± 0%), and old castrated females (0 ± 0 and 1 ± 0%).

DISCUSSION

The aging glomerulus develops a slowly evolving injury that is particularly pronounced in the rat (1–3). The mechanism of this progressive glomerular disease is unknown; however, it has been suggested that high glomerular capillary blood pressure plays a primary role in this, as in other progressive glomerular diseases (1). Recent studies by us, however, have indicated that age-dependent glomerular injury is not due to glomerular hypertension (12,13). The accumulation of glomerular extracellular matrix material has been suggested to provide a primary stimulus to the eventual development of glomerular sclerosis in a variety of renal diseases including aging (3,4). The quantity of the extracellular matrix of the glomerular basement membrane and mesangium is controlled by a balance between synthesis and specific enzymatic degradation processes. So far, three glomerular metalloproteases have been described in glomerular homogenates and in primary cultures of glomerular epithelial and mesangial cells (6–8). The glomerular epithelial and mesangial metalloproteases are both secreted, and the latter is inhibited by an endogenous tissue inhibitor of metalloprotease (TIMP) (6,8). There is also a recently described membrane-bound metalloprotease that is not inhibited by TIMP (7). All of these glomerular metalloproteases hydrolyze type IV collagen (the major collagen subtype in the basement membrane and the mesangial matrix) at neutral pH, suggesting that these metalloproteases are important in the normal turnover of mesangial matrix and glomerular basement membrane.

Accordingly, in this study, we investigated whether aging produced reductions in glomerular metalloprotease activity that might lead to matrix expansion and ultimately to age-dependent glomerular injury. In intact males, substantial age-dependent glomerular injury is evident by 20 months of age; however, there is no change in glomerular metalloprotease activity in old versus young male rats, who exhibit little glomerular damage. This observation suggests that matrix material degradation proceeds at a constant rate in the old rat but that there may be an enhanced stimulus to matrix production with advancing age. Alternatively, the glomerular metalloprotease activity in the old intact male may be inhibited by age-related changes in the matrix, such as nonenzymatic glycosylation, making the matrix inaccessible to degradation by the glomerular metalloproteases (14), or by age-related increases in the production of endogenous inhibitory substances such as TIMP (8).

Our observations in females and castrated rats of both sexes suggest that glomerular metalloprotease activity is important in protecting the glomerulus from age-dependent injury. In young females, glomerular metalloprotease activity is low and similar to that seen in young and old intact males; however, glomerular metalloprotease activity doubles with advancing age in intact females. Similarly elevated levels of glomerular metalloprotease activity are seen in old ovariectomized females and in old castrated males. Our studies do not rule out the possibility that in females and castrated rats the glomerular metalloprotease that increases with advancing age may be that responsible for the degradation of type I rather than type IV collagen. Alternatively, there may be an age-dependent decrease in TIMP activity in females and castrated rats. However, regardless of the mechanism, these three groups show conservation of glomerular structure with little glomerular injury by 20 months of age. Thus, an age-dependent increase in glomerular metalloprotease activity may be an essential protective mechanism that prevents the build-up of matrix proteins and protects against age-dependent glomerular injury.

The sex difference in the vulnerability to the development of glomerular injury (males more susceptible) is not hemodynamically mediated, as shown by us previously (12,13). The observations presented
here raise the possibility that increased matrix accumulation in the old male rat might contribute to the sex difference in glomerular injury in response to aging. The male rat is also much more susceptible to the development of glomerular sclerosis after subtotal nephrectomy (15), and mesangial expansion and glomerular procollagen (α1 IV) mRNA levels were higher in nephrectomized males compared with nephrectomized females. Thus, in this model of rapidly progressing glomerular sclerosis, the male is more susceptible both to glomerular injury and to increased matrix production. This enhanced susceptibility to the development of glomerular injury is apparently related to the presence of the androgens, because castration of the male protects against the development of glomerular injury due to uninephrectomy (16). Our studies also suggest that the presence of testosterone provides the risk factor for age-dependent glomerular damage because the glomerular architecture was similarly preserved in castrated males and in intact and ovariectomized females. Thus, castration of the male was beneficial in protection against age-dependent glomerular injury.

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REFERENCES