C6 Mediates Chronic Progression of Tubulointerstitial Damage in Rats with Remnant Kidneys

MASAOMI NANGAKU,* JEFFREY PIPPIN,† and WILLIAM G. COUSER‡
*Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, Tokyo, Japan, and ‡Division of Nephrology, University of Washington Medical Center, Seattle, Washington.

Abstract. Although it was once considered only a marker of glomerular damage, accumulating evidence indicates that proteinuria per se is nephrotoxic and contributes to the progression of renal injury. Several studies have demonstrated that activation of complement in proteinuric urine results in tubular and interstitial damage. It was previously demonstrated that acute complement-mediated interstitial disease is induced by C5b-9. Here the role of C5b-9 in the progression of chronic proteinuric renal disease was investigated in a nonimmunologic remnant kidney model. Five-sixths nephrectomies were performed for normocomplementemic control and C6-deficient PVG rats. Tubulointerstitial injury was assessed by measurement of two independent markers of tubular injury (i.e., vimentin and osteopontin), interstitial accumulation of the extracellular matrix components collagen type I, collagen type IV, and laminin, interstitial macrophage infiltration, and renal function. The two groups developed similar levels of proteinuria and BP. Whereas C3 deposition on the brush border was equivalent for rats in the two groups, C5b-9 deposition was observed only for normocomplementemic rats. At day 35, the degrees of both tubulointerstitial injury and renal failure were the same for the two groups. Tubulointerstitial injury in normocomplementemic rats was still severe at day 70. In contrast, interstitial injury in C6-deficient rats had improved markedly at day 70, with improvements in renal function. In a rat model of chronic progressive renal disease secondary to nephron loss, the initial interstitial changes are complement-independent and largely reversible, whereas progressive interstitial fibrosis is mediated predominantly by C5b-9. Treatment to reduce C5b-9 attack in tubular cells may slow progression and facilitate recovery.

The relationship between proteinuria and poor prognoses in kidney disease has been well established in several large-scale studies (1,2). Although proteinuria had been considered merely a marker or consequence of glomerular damage, many recent studies indicate that it may be a cause of progressive renal injury (reviewed in references 3–6). However, the mechanisms by which increased urinary protein concentrations are toxic to the kidney are poorly understood.

Tubulointerstitial injury is observed for most patients with nonselective glomerular proteinuria. Since the original observation by Risdon et al. (7), which was further developed by other authors (8–12), it has been known that the extent of tubulointerstitial damage is better correlated with the impairment of renal function than is the degree of glomerular damage.

Tubulointerstitial injury leads to deterioration of renal function via several different mechanisms. Tubular atrophy in-creases fluid delivery to the macula densa and triggers reductions in GFR via tubuloglomerular feedback (13). It also results in obliteration of postglomerular capillaries, leading to ischemic renal injury and increased tubulointerstitial backleakage through denuded tubular basal membranes. Tubular damage also leads to atubular glomeruli and decreases the number of functional nephrons (14,15).

Accumulating evidence now suggests a link between heavy proteinuria, subsequent tubulointerstitial injury, and eventual kidney failure (3,16). Several mechanisms likely participate, including direct injury to intracellular lysosomal pathways and lysosomal rupture attributable to reabsorbed proteins (17), filtration and reabsorption of a lipid-bound chemotactic factor (18), oxidative injury induced by transferrin reabsorption (19), and intratubular complement activation, leading to tubular cell activation or injury (20,21).

We recently provided evidence, using the aminonucleoside-induced nephrosis model of the nephrotic syndrome, that complement-induced interstitial damage is mediated by sublytic C5b-9 attack on proximal tubular cells (22). In this study, we extend these observations to demonstrate that C5b-9 is the principal mediator of progressive interstitial damage in a model of progressive renal disease secondary to reduced renal mass, rather than primary glomerular injury. In the absence of C6, acute interstitial changes resolve despite ongoing proteinuria.

Materials and Methods

Animals

Male complement-sufficient PVG rats were originally obtained from Harlan Sprague-Dawley (Cambridge, UK), and C6-deficient PVG rats were obtained from Bantin and Kingman Univers (Free-
of the total area that was positively stained was calculated by using the NIH Image program for image analysis, in a blinded manner.

Histologic Assessment of Glomerulosclerosis
PAS-stained sections from each rat were studied histologically for glomerulosclerosis, which was defined as glomeruli exhibiting evidence of segmental or global collapse of capillaries, with or without associated hyaline deposition and adhesion of the capillary tuft to Bowman’s capsule. The extent of glomerulosclerosis was expressed as a percentage of the total number of glomeruli counted (>100/animal).

Estimation of the Number of Glomeruli
PAS-stained sections obtained from each rat before induction of disease were studied histologically for estimation of the number of glomeruli. Glomeruli were counted in 10 randomly selected cortical fields with a ×10 objective, in a blinded manner. Age- and gender-matched, untreated, normocomplementemic and C6-deficient rats were euthanized for measurement of kidney weights.

Statistical Analyses
Data are reported as mean ± SD. Statistical comparisons were performed with the program StatView (Abacus Concepts, Berkeley, CA), using ANOVA followed by the Bonferroni-Dunn method for multiple-group comparisons. Nonparametric data were analyzed with the Kruskal-Wallis test when appropriate. Differences with P values of <0.05 were considered significant.

Results
Proteinuria and BP
The amounts of proteinuria in both groups increased continuously throughout the experimental period and peaked at week 10 (Table 1). There were no significant differences between the two groups, indicating a lack of effect of C5b-9 on the degree of hemodynamic glomerular injury. BP values measured at week 10 did not differ between C6-sufficient and C6-deficient rats (average systolic BP, 131 ± 21 and 135 ± 16 mmHg, respectively; P = 0.69).

PAS Staining
Histologic assessments of tubulointerstitial injury were performed with PAS staining. The numbers of tubules with dilation and/or degeneration, loss of the brush border, and detachment of tubular epithelial cells from the basement membrane were similar in the two groups at day 35 (C6-sufficient rats, 2.4 ± 1.7; C6-deficient rats, 2.5 ± 1.8; P = 0.85). However, whereas tubulointerstitial injury in the C6-deficient rats was resolved at day 70, compared with day 35 (0.2 ± 0.5 versus 2.5 ± 1.8, P < 0.05), that in C6-sufficient rats was maintained (2.5 ± 1.3 versus 2.5 ± 1.8, P > 0.05) (Figure 1).

Vimentin Staining
Vimentin staining was performed for assessment of tubulointerstitial injury (Figure 2, a through d). The numbers of vimentin-positive tubules and tubules surrounded by vimentin-positive cells did not differ between the two groups at day 35.
Table 1. Urinary protein excretion and serum creatinine levels in C6-sufficient and C6-deficient rats with remnant kidneys

<table>
<thead>
<tr>
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<th>Proteinuria (mg/d)</th>
<th>Creatinine levels (mg/dl)</th>
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<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>C6-sufficient (n = 9)</td>
<td>11.2 ± 3.0</td>
<td>18.4 ± 9.3</td>
</tr>
<tr>
<td>C6-deficient (n = 5)</td>
<td>10.5 ± 1.6</td>
<td>15.4 ± 3.9</td>
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Figure 1. Light micrographs of remnant kidneys from normocomplementemic (A and C) and C6-deficient (B and D) PVG rats. Both normocomplementemic and C6-deficient PVG rats exhibited severe tubulointerstitial injury at day 35 (A and B). At day 70, however, tubulointerstitial damage in C6-deficient rats had markedly improved (D), whereas that in normocomplementemic rats had increased in severity (C). Magnification, ×200.

(Table 2). Fiftyfold more vimentin-positive tubules and tubules surrounded by vimentin-positive cells were observed in the cortex for the C6-sufficient rats, compared with the C6-deficient rats, at day 70. In fact, vimentin-positive tubules had largely disappeared by day 70 among C6-deficient animals, despite ongoing proteinuria (Table 2). Staining for vimentin in glomeruli was identical for the two groups at both time points.

Osteopontin Staining

Osteopontin was used as another marker of tubular injury (25) (Figure 2, e through h). The numbers of osteopontin-positive tubules were not different between the two groups at day 35 (Table 2). However, >15-fold more osteopontin-positive tubules were observed in the cortex for the C6-sufficient rats at day 70, whereas osteopontin staining had almost disappeared in the tubules of the C6-deficient rats at the same time point (Table 2).

PCNA-Positive Tubular Cells

We also performed PCNA staining to assess the proliferation of tubular cells as a consequence of C5b-9-induced tubular damage. The number of PCNA-positive tubular cells was
smaller in C6-deficient rats than in normocomplementemic rats, both at day 35 (complement-sufficient rats, 6.5 ± 5.5 PCNA-positive tubular cells per cortical field, complement-deficient rats, 3.8 ± 2.1; \( P = 0.25 \)) and at day 70 (complement-sufficient rats, 4.4 ± 4.3; complement-deficient rats, 1.4 ± 0.5). However, these differences did not reach statistical significance (\( P = 0.25 \) at day 35 and \( P = 0.22 \) at day 70).

**Extracellular Matrix Accumulation**

To evaluate extracellular matrix accumulation, we performed quantitative image analysis of staining for collagen IV (Figure 3, a through d), laminin (Figure 3, e through h), and collagen I (Figure 3, i through l). The amounts of these matrix components increased at day 35 (Table 3). Staining for these matrix components in C6-deficient rats was decreased at day 70, compared with day 35, whereas that in complement-sufficient rats remained high (fibronectin) or increased (collagen I and IV) (Table 3). Although the amount of collagen I in C6-deficient rats was increased at day 70, compared with day 35, the difference did not reach statistical significance (\( P = 0.27 \)).

**Macrophage Infiltration**

The numbers of infiltrating macrophages in the tubulointerstitial were similar for the two groups at day 35 (complement-sufficient rats, 59.6 ± 58.6 ED-1-positive cells per cortical field). However, these differences did not reach statistical significance (\( P = 0.25 \) at day 35 and \( P = 0.22 \) at day 70).

![Figure 2](image)
field; complement-deficient rats, 49.4 ± 34.7; \( P = 0.73 \)). However, at day 70, the number of macrophages in the tubulointerstitium in C6-deficient animals was decreased (10.4 ± 7 versus 49.4 ± 34.7, \( P < 0.05 \)), whereas that in complement-sufficient animals remained high (59.6 ± 58.6 versus 53.1 ± 47.9, \( P > 0.05 \)).

**Renal Function**

To estimate renal function, we measured serum creatinine levels (Table 1). The two groups demonstrated similar elevations in serum creatinine levels, compared with baseline values, at 2 wk. At 5 wk, serum creatinine levels had returned to near-baseline values for both groups. However, elevations were observed again at day 70 for both groups, and renal function was significantly worse for the C6-sufficient group (Table 1).

**C3 and C5b-9 Deposition in Tubules**

C5b-9 formation was detected only on the proximal tubular brush borders of complement-sufficient rats at both day 35 and day 70, whereas C6-deficient animals exhibited negative staining for C5b-9 at both times points (Figure 4A). C3 deposition was also observed on the tubular brush borders, but there was no difference between complement-sufficient and C6-deficient animals (Figure 4B).

**Glomerulosclerosis**

To investigate whether a difference in glomerular changes could account for differences in interstitial disease, we evaluated glomerulosclerosis in the experimental animals at day 70. Whereas 4.3 ± 2.8% of glomeruli in C6-deficient rats developed sclerosis, 10.9 ± 13.9% of glomeruli in complement-sufficient rats demonstrated sclerotic changes. The difference did not reach statistical significance (\( P = 0.32 \)).

**Glomerular Density**

Because renal injury in remnant kidneys is a consequence of reduced nephron mass, we evaluated whether the different responses to renal ablation demonstrated by normocomplementemic and C6-deficient rats could be attributed to differences in the numbers of glomeruli in these strains. Among healthy animals, kidney weights did not differ between normocomplementemic rats (963 ± 11 mg, \( n = 4 \)) and C6-deficient rats (981 ± 22 mg, \( n = 10 \)) (\( P = 0.64 \)). We also counted the number of glomeruli per section. The number of glomeruli did not differ between the two strains (normocomplementemic rats, 18.1 ± 4.2 glomeruli/low-power field; C6-deficient rats, 19.5 ± 3.2 glomeruli/low-power field; \( P = 0.52 \)).

**Discussion**

The rat remnant kidney model has been widely used to identify mechanisms responsible for the progression of renal disease. Glomerular capillary hypertension, exposure of endothelial cells to shear stress and of mesangial cells to cyclical stretching, direct effects of angiotensin II on mesangial cells, and subsequent upregulation of transforming growth factor-\( \beta \) have been proposed as critical mediators of glomerular injury in this model (26,27).

Although many investigators have extensively studied glomerular sclerosis in remnant kidneys, only a few studies have focused on the tubulointerstitial damage in this model (15,28–31). Among those studies, that by Abbate et al. (31) is of particular importance to our work, because those authors localized C3 staining in the brush borders of tubules subjected to a high filtered protein load, with surrounding inflammatory cells, consistent with a pathogenic role for urinary complement activation of tubular cells in this model. Furthermore, those authors extended their studies to report renoprotective effects of treatment with an angiotensin-converting enzyme inhibitor, presumably via limitation of proteinuria and thus tubular activation of complement (30).

Recent studies have also focused on the importance of complement components in proteinuric urine as mediators of tubulointerstitial injury (32,33). Complement-depletion studies by Matsuo and colleagues (34,35) and by our group (22), using acute proteinuric models of glomerular injury, provided more direct evidence for deleterious effects of urinary complement components on the tubulointerstitium in a relatively short period (until day 14). We also demonstrated protective effects of an endogenous complement regulatory protein (Crry) against the harmful effects of tubular complement activation in the setting of massive proteinuria (36).

In the pursuit of a better understanding of progressive renal disease, both the development of glomerulosclerosis and interstitial fibrosis consequent to reduced nephron mass and the effects of proteinuria on the tubulointerstitium have been extensively studied by many laboratories, including our own. However, two observations that are new and of considerable potential significance emerge from this study of the remnant kidney model in C6-deficient PVG rats.

The first observation is that, at least in this rat strain, there seem to be two phases of interstitial disease, rather than one continuous progressive one. The first phase occurs early, up to approximately 1 mo, and seems to be reversible. It is indicated by the dramatic resolution of tubular injury in C6-deficient
Figure 3. Photomicrographs demonstrating the accumulation of extracellular matrix in normocomplementemic (a, c, e, g, i, and k) and C6-deficient (b, d, f, h, j, and l) PVG rats with remnant kidneys (a to d, collagen IV; e to h, laminin; i to l, collagen I). Immunohistochemical analyses were performed for collagen type IV, laminin, and collagen type I. Both normocomplementemic and C6-deficient rats demonstrated marked deposition of collagen IV (a and b), laminin (e and f), and collagen I (i and j) at day 35 (D35). Deposition of those extracellular matrix components improved at day 70 (D70) in C6-deficient rats (d, h, and l), whereas accumulation of those components was still severe in normocomplementemic rats at that time point (c, g, and k). Magnification, ×200.
animals between days 35 and 70, as illustrated by marked reductions in gross histologic injury, markers of tubular damage, macrophage infiltrates, and even, to a lesser extent, interstitial scarring. Although renal function, as indicated by serum creatinine levels, did not parallel these changes, progression was also reduced in C6-deficient animals, compared with C6-sufficient control animals. We are unaware of any previous description of this phenomenon, which suggests that the early interstitial changes likely reflect effects of the abrupt increases in glomerular, and likely interstitial, pressures and flows after 5/6 nephrectomy, rather than being mediated by proteinuria, which is not present at that time. Glomerular release of a variety of cytokines and inflammatory mediators as a consequence of stretch, sheer stress, increased angiotensin II levels, and other acute effects can readily be envisioned. After these new adaptive hemodynamic conditions are in place, however, these events seem to resolve.

The second observation, of potentially more significance, is that, between 35 and 70 d (the period during which substantial proteinuria did develop), progressive interstitial injury was maintained only in normocomplementemic animals, despite the fact that equivalent degrees of proteinuria were present in the two groups. Although complement (presumably primarily C5b-9) has previously been considered only one of several factors that mediate interstitial injury in proteinuric states, as discussed above, presumably the non-complement-related factors were equally present in the two groups. However, only the complement-sufficient animals exhibited persistent disease, whereas the C6-deficient animals demonstrated remarkable resolution of fibrosis and markers of tubular injury between days 35 and 70. These findings indicate that C5b-9 is not just one of several tubulotoxic factors that occur in proteinuric disorders but is in fact the principal one. If this is correct, then therapy to prevent intratubular complement activation might well be more effective than therapy directed against any proposed mediators of progression other than the proteinuria itself.

We cannot exclude the possibility that the susceptibility of

Table 3. Quantification of immunohistochemical staining for collagen I, collagen IV, and laminin before the operation and at weeks 5 and 10 after 5/6 nephrectomy

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Day 35</th>
<th>Day 70</th>
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<tbody>
<tr>
<td>Collagen I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6-sufficient</td>
<td>Not done</td>
<td>10.3 ± 7.7</td>
<td>46.0 ± 22.1</td>
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<tr>
<td>(n = 9)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C6-deficient</td>
<td>Not done</td>
<td>13.7 ± 7.2</td>
<td>20.3 ± 9.6</td>
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<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.41</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Collagen IV</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C6-sufficient</td>
<td>7.4 ± 1.4</td>
<td>24.3 ± 6.1</td>
<td>29.0 ± 14.9</td>
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<tr>
<td>(n = 9)</td>
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<tr>
<td>C6-deficient</td>
<td>7.1 ± 2.1</td>
<td>18.9 ± 3.8</td>
<td>14.1 ± 3.6</td>
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<tr>
<td>(n = 5)</td>
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<td></td>
<td></td>
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<tr>
<td>P value</td>
<td>0.94</td>
<td>0.21</td>
<td>&lt;0.005</td>
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<tr>
<td>Laminin</td>
<td></td>
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<tr>
<td>C6-sufficient</td>
<td>4.6 ± 1.7</td>
<td>17.0 ± 10.3</td>
<td>16.1 ± 5.0</td>
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<tr>
<td>(n = 9)</td>
<td></td>
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<td></td>
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<tr>
<td>C6-deficient</td>
<td>4.9 ± 1.9</td>
<td>17.0 ± 10.7</td>
<td>5.9 ± 1.4</td>
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<tr>
<td>(n = 5)</td>
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<tr>
<td>P value</td>
<td>0.93</td>
<td>1.00</td>
<td>&lt;0.05</td>
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Figure 4. Photomicrographs demonstrating that, whereas C3 deposition on tubular cells was the same in the two groups, C5b-9 formation was observed only in normocomplementemic rats. Photomicrographs demonstrate the deposition of C5b-9 (A) and C3 (B) in C6-deficient (b and d) and C6-sufficient (a and c) PVG rats. C3 staining was equivalent in normocomplementemic and C6-deficient PVG rats at day 35 (D35) (B, a and b) and day 70 (D70) (B, c and d). In contrast, C5b-9 formation was observed only in normocomplementemic rats at day 35 (A, a) and day 70 (A, c); no staining for C5b-9 was detected in C6-deficient rats at either time point (A, b and d). Arrows, deposition of complement products.
PVG rats to interstitial disease may be different from that of more commonly used strains such as Sprague-Dawley. Previous studies of PVG rats suggested that these rats exhibit reduced susceptibility to fibrosis in the form of glomerulosclerosis induced by uninephrectomy (37). However, this difference in fibrosis was in comparison with other rat strains that developed more sclerosis and more proteinuria. In our study, both C6-sufficient and C6-deficient animals had the same PVG background, with equivalent amounts of proteinuria; therefore, these strain differences cannot explain our results. The remnant kidney model produced by surgical excision generally exhibits a relatively mild course, without marked hypertension (38). This may explain why some of our histologic assessments (vimentin, osteopontin, and laminin staining assays) failed to demonstrate progression of tubulointerstitial damage in normocomplementemic rats at day 70. In our study, experimental and control rats were all PVG rats and were equally proteinuric. Therefore, whatever differences may exist between PVG rats and other strains are not relevant to this study, in which the only known difference between the experimental and control groups was C6 deficiency. We confirmed that there was no difference in nephron numbers between normocomplementemic and C6-deficient PVG rats in this study. We were unable to identify any other difference between complement-sufficient and C6-deficient rats in this study and many other previous studies from our laboratory (22,23,39), and we think that it is fair to identify the inability to form C5b-9 and deposit it in tubules, which was clearly significantly different between the two groups, as the principal (albeit not only) mechanism involved.

The resolution of injury observed in C6-deficient rats is of great interest. The exact mechanism by which tubulointerstitial injury heals in this model remains to be elucidated. Regardless of mechanisms, however, our findings suggest that the role of C5b-9 in mediating acute tubular injury is less than that of other early reactants but C5b-9 is more important in the chronic progressive phase of interstitial disease.

Hostetter and colleagues (40–42) demonstrated that complement components in nonselective proteinuria are activated on the apical membrane of proximal tubules, with protein overload in the absence of antibody deposition. Moreover, tubular cells injured via any mechanism may become alternative pathway activators (43). There are probably multiple mechanisms by which C5b-9 leads to tubular and interstitial injury (44–46).

In summary, these studies confirm and extend previous observations on the role of complement in the tubulointerstitial damage that occurs in the setting of heavy nonselective proteinuria. Our study is the first to demonstrate that C5b-9 mediates continuous tubulointerstitial damage in a chronic proteinuric model. We also demonstrate marked improvement of early tubulointerstitial injury in the remnant kidney when the ability to form C5b-9 is abrogated. These findings suggest that sublytic tubular C5b-9 attack consequent to proteinuria is the most important contributing factor in inducing tubulointerstitial damage and progressive loss of nephron function in chronic proteinuric kidney diseases, which occur as a consequence of decreases in the number of functioning nephrons, with hemodynamic adaptations in the remainder (e.g., most progressive renal diseases). Prevention of C5b-9 formation in chronic progressive renal disease may slow or prevent progression and facilitate recovery of renal function.

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