Complement Membrane Attack Complex (C5b-9) Mediates Interstitial Disease in Experimental Nephrotic Syndrome

MASAOMI NANGAKU, JEFF PIPPIN, and WILLIAM G. COUSER
Division of Nephrology, Department of Medicine, University of Washington, Seattle, Washington.

Abstract. Accumulating evidence suggests that the generation of complement activation products from filtered complement components in urine with nonselective proteinuria leads to tubulointerstitial disease, resulting in progressive loss of renal function. To elucidate the role of C5b-9 in complement-mediated effects on renal tubular cells exposed to proteinuric urine, equivalent levels of proteinuria were induced (using the amino-nucleoside of puromycin) in normocomplementemic and genetically C6-deficient piebald viral glaxo (PVG) rats. Semi-quantitative histologic analysis revealed that complement-sufficient animals developed more severe tubulointerstitial disease than did C6-deficient rats. Amelioration of tubulointerstitial damage in C6-deficient animals was confirmed by studies with three independent markers of tubular damage, i.e., vimentin, osteopontin, and proliferating cell nuclear antigen. More tubular epithelial cells expressed osteopontin (an early marker of tubular injury) in normocomplementemic rats, compared with C6-deficient rats, at both days 7 and 12. Staining of vimentin in the tubules, near areas of tubular damage, was increased in normocomplementemic rats at day 12, and more proliferating cell nuclear antigen-positive tubular cells were observed at day 12 in complement-sufficient animals. The tubulointerstitial damage in complement-sufficient rats was also associated with greater accumulation of extracellular matrix (fibronectin) at day 12. These studies document for the first time an important role for C6, and therefore C5b-9, in the pathogenesis of non-immunologic tubulointerstitial injury induced by proteinuria. These findings suggest that C5b-9 formation resulting from proteinuria contributes to the loss of nephron function by damaging the tubulointerstitium and that prevention of C5b-9 formation in tubules could slow the deterioration of renal function.

Considerable evidence from both clinical and experimental studies suggests that progressive loss of renal function in various glomerular diseases is better correlated with structural damage in the renal interstitium than with that in the glomeruli (1–8). The mechanisms by which tubulointerstitial injury leads to loss of renal function and reduction of GFR are likely multifactorial. Tubular atrophy may trigger reduction of GFR through tubuloglomerular feedback by increasing fluid delivery to the macula densa (5). Interstitial fibrosis may occlude postglomerular capillaries and reduce glomerular blood flow (5). Tubular damage may also lead to atubular glomeruli and may decrease the number of functional nephrons (9,10). However, the factors most compelling that correlated with interstitial changes in glomerular disease, particularly in human subjects, are the level and duration of urine protein excretion (11–14).

Several hypotheses have been advanced to account for the apparent role of increased glomerular protein filtration in causing interstitial inflammation (reviewed in references (14 through 18). High protein concentrations in tubular fluid can themselves be toxic to tubular cells (19). Tubular cells reabsorbing lipid bound to filtered albumin can generate lipid-derived chemotactic factors (20,21). Generation of free iron from filtered transferrin can lead to oxidant-mediated tubular and interstitial damage (22,23).

However, another possible link between nonselective proteinuria and tubulointerstitial disease involves the generation of products of complement activation from filtered individual complement components, through amidation of C3 to form a convertase (24,25) or through the effects of damaged renal cells, which may become direct activators of the complement cascade (26). This hypothesis was initially put forth by Nath et al. (27), who documented the ability of sodium bicarbonate administration to reduce C3 activation, tubular C3 and C5b-9 deposition, and interstitial disease in the proteinuric remnant kidney model in rats. Similar tubular and interstitial complement deposits have been well described in a variety of other proteinuric disorders in both experimental animals and human subjects (28,29). Although the exact mechanism of this apparently complement-mediated interstitial disease has not been defined, correlations between tubular C5b-9 deposits and interstitial inflammation and fibrosis have been documented in IgA nephropathy (30), lupus nephritis (31), and a variety of other proteinuric human glomerular diseases (28).

Stimulated by the observations outlined above, we tested the hypothesis that C5b-9-mediated effects on renal tubular cells might account for the interstitial inflammation that accompanies most proteinuric disorders. C5b-9 formation on glomerular cells has been well documented to cause glomerular injury (32–35). We tested this hypothesis by inducing equivalent
levels of proteinuria, using the aminonucleoside of puromycin, in normocomplementemic and genetically C6-deficient piebald viral glaxo (PVG) rats. Our findings document an important role for C6, and therefore C5b-9, in the pathogenesis of interstitial disease in this non-immunologically mediated proteinuric model.

**Materials and Methods**

**Animals**

Three-month-old male PVG rats weighing 250 to 300 g were used for the experiments. Two vendors provided PVG rats of different complement status. Complement-deficient PVG rats were obtained from Bantin and Kingman Universal (Edmonds, WA). Age- and gender-matched PVG rats with normal complement activity were obtained from Harlan Sprague Dawley (Cambridge, United Kingdom). Before study, the hemolytic activity in serum from each rat was measured using a standard 50% complement hemolytic (CH50) assay (36). All rats from Harlan Sprague Dawley exhibited normal CH50 levels. All rats from Bantin and Kingman Universal exhibited <1% of normal CH50 activity. A detailed analysis of levels of individual complement components in complement-deficient PVG rats has been published elsewhere (36). All rats were housed in individual cages in a temperature- and light-controlled environment in an accredited animal care facility, with free access to food and water. All studies conform to current guidelines presented in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Induction of Puromycin Aminonucleoside Nephrosis**

Aminonucleoside-induced nephrosis was produced by intravenous administration of the aminonucleoside of puromycin (100 mg/kg; ICM Pharmaceuticals, Life Sciences Group, Cleveland, OH). On days 7 and 12 after puromycin aminonucleoside administration, a 24-h urine sample was collected for measurement of protein excretion, using individual metabolic cages providing free access to water. After completion of urine collections, rats underwent a survival (day 7) (normocomplementemic, n = 8; C6-deficient, n = 7) or sacrificial (day 12) (normocomplementemic, n = 5; C6-deficient, n = 7) renal biopsy under ether anesthesia.

**Renal Histologic and Immunohistochemical Analyses**

Tissue fixed in methyl Carnoy’s fixative was processed, and 4-μm sections were stained with the following antibodies. Osteopontin was localized using an indirect immunoperoxidase method with a polyclonal goat anti-osteopontin antibody (a generous gift from Ceci Giachelli, University of Washington, Seattle, WA). Vimentin was identified using the murine monoclonal IgG antibody V9 (Dako, Carpinteria, CA). Proliferating cells were identified using 19A2, a monoclonal IgM antibody to proliferating cell nuclear antigen (PCNA) (Coulter Immunology, Hialeah, FL). Tissue for immunofluorescence analysis was embedded in OCT compound (Lab-Tek Products, Miles Laboratories, Naperville, IL) and snap-frozen in liquid nitrogen-cooled isopentane. Rat C3 was detected with FITC-conjugated goat anti-rat C3 (ICN Pharmaceuticals, Irvine, CA). The presence of rat C5b-9 was determined using biotinylated anti-rat C5b-9 monoclonal antibody 2A1 (37), followed by FITC-streptavidin.

**Quantification of Tubulointerstitial Disease**

Tubulointerstitial injury was scored semiquantitatively, using a ×20 objective, as described previously (38). Thirty cortical fields of periodic acid-Schiff-stained biopsies were assessed in a blinded manner. Tubulointerstitial injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane and was scored on a scale of 0 to 4, as follows: 0, no tubulointerstitial injury; 1, <25% of the tubulointerstitium injured; 2, 25 to 50% of the tubulointerstitium injured; 3, 51 to 75% of the tubulointerstitium injured; 4, >75% of the tubulointerstitium injured.

The number of PCNA-positive tubular cells was expressed as the mean ± SD of the number per high-power field. This value was calculated from counts obtained for 25 randomly selected cortical interstitial areas for each rat kidney, in midcortical sections, in a blinded manner.

**Optimas Quantification of Osteopontin and Fibronectin**

Using computer-assisted image analysis software (Optimas, version 6.2; Media Cybernetics, Silver Spring, MD), the percent area occupied by osteopontin-positive and fibronectin-positive tubules (including the entire cortical area except glomeruli) was measured for each field at ×50 magnification; a quantitative measurement of the mean percent areas for 20 fields was then calculated for each biopsy, from digitized images. The mean percent areas for individual biopsies were then averaged to yield a mean percent area for each group of animals (39).

**Serum CH50 and Urinary Protein Measurements**

The hemolytic activity of complement was measured as the hemolysis of sheep erythrocytes sensitized by specific antibodies (anti-sheep hemolysin) (40). The degree of hemolysis was expressed in CH50 units. One CH50 unit is defined as the volume of serum that lyses 50% of the erythrocytes in the reaction mixture. The number of CH50 units in 1 ml of serum is the hemolytic titer. Urine protein excretion was measured using the sulfosalicylic acid method.

**Statistical Analyses**

Data were reported as mean ± SD. Data were analyzed by t test, with adjustments using the Bonferroni/Dunn method.

**Results**

**Urinary Protein Excretion Did Not Differ Between the Two Groups**

Administration of puromycin induced proteinuria in both normocomplementemic and C6-deficient animals. Urinary protein excretion was not different between the two groups of rats on either day 7 (complement-sufficient, 88.4 ± 8.20 mg/d; C6-deficient, 98.0 ± 10.6 mg/d; P = 0.48) or day 12 (complement-sufficient, 140 ± 16.6 mg/d; C6-deficient, 124 ± 12.8 mg/d; P = 0.19). The equivalent levels of proteinuria in the two groups of rats enabled us to examine the specific effects of C6 deficiency on the tubulointerstitial lesions that accompany proteinuria.

**C3 and C5b-9 Staining Confirmed C6 Deficiency in Complement-Deficient Rats**

Strongly positive staining for C5b-9 was observed only on the proximal tubular brush borders of complement-sufficient PVG rats, whereas C6-deficient animals exhibited negative
results (Figure 1). C3 deposition was also seen on tubular brush borders, but there was no difference in C3 staining between complement-sufficient and C6-deficient animals (data not shown).

Complement-Sufficient Rats Demonstrated More Severe Tubulointerstitial Damage

To compare the effects of C6 deficiency on tubulointerstitial damage, we performed a semiquantitative histologic analysis

Figure 1. C5b-9 deposition in tubules. C5b-9 formation was observed only at the apical membrane of renal tubules from normocomplementemic rats (A). In contrast, C6-deficient rats showed no staining with anti-C5b-9 monoclonal antibody (B). The staining was very faint, and the photograph is overexposed to show the nonspecific background staining of the tissues. Magnification, ×400.

Figure 2. Light micrographs for normocomplementemic (A and B) and C6-deficient (C and D) PVG rats at days 7 (A and C) and 12 (B and D). Normocomplementemic animals exhibited a diffuse increase in mononuclear cell infiltration throughout the interstitium at day 7 (A), which was increased and accompanied by increased matrix deposition at day 12 (B). Both cellular infiltration and matrix expansion were significantly reduced in C6-deficient animals at days 7 (C) and 12 (D). Periodic acid-Schiff staining. Magnification, ×200.
of tubulointerstitial injury, combining tubular dilation, atrophy, cast formation, cell sloughing, and basement membrane thickening into a single score (Figure 2). Our studies revealed more severe tubulointerstitial injury in normocomplementemic rats than in complement-deficient rats at day 7 (0.59 ± 0.12 versus 0.33 ± 0.12), although this difference did not reach statistical significance (P = 0.17). However, complement-sufficient rats developed significantly more tubulointerstitial injury by day 12, compared with C6-deficient animals (1.98 ± 0.047 and 0.81 ± 0.21, respectively; P < 0.005).

Vimentin Was Upregulated in Complement-Sufficient Rats at Day 12
To confirm the difference in tubulointerstitial injury observed in the histologic analysis described above, we performed immunohistochemical studies using three different markers of tubular injury, i.e., vimentin, osteopontin, and PCNA. Staining for vimentin in the tubules of the complement-sufficient rats at day 7 and in those of the C6-deficient animals at any time point yielded negative results. We observed vimentin-positive tubules only in normocomplementemic rats and only at day 12 (Figure 3).

Osteopontin Was Upregulated in Complement-Sufficient Rats at Days 7 and 12
Osteopontin scores were estimated as percent areas using Optimas software. At day 7, complement-sufficient rats showed more osteopontin-positive tubules than did C6-deficient rats (3.42 ± 0.91 and 0.96 ± 0.32% area, respectively; P < 0.0005). Osteopontin expression was increased at day 12 for both groups, but the difference was still significant, with much more osteopontin expression in normocomplementemic animals (13.7 ± 2.3 versus 7.98 ± 1.13% area; P < 0.05) (Figure 4).

More Proliferating Tubular Cells Were Observed in Complement-Sufficient Rats
At day 7, there was no difference in the number of PCNA-positive tubular cells between complement-sufficient and complement-deficient rats (0.40 ± 0.10 and 0.27 ± 0.08/high-power field, respectively; P > 0.05). However, at day 12, more PCNA-positive tubular cells were observed in normocomplementemic rats than in C6-deficient rats (1.05 ± 0.23 and 0.41 ± 0.06/high-power field, respectively; P < 0.05) (Figure 5).

Figure 3. Photomicrographs demonstrating the deposition of vimentin intermediate filaments in normocomplementemic (A and B) and C6-deficient (C and D) PVG rats at days 7 (A and C) and 12 (B and D). At day 7, kidneys showed reactivity with anti-vimentin antibody in glomeruli and peritubular capillaries, whereas tubular cells always yielded negative results for the two groups of rats. However, tubules from normocomplementemic rats showed increased staining for vimentin near areas of tubular damage at day 12. Magnification, ×200.
Extracellular Matrix Accumulation Was More Severe in Complement-Sufficient Rats

We also quantified accumulation of a representative extracellular matrix protein, fibronectin, at day 12. Quantification using Optimas software demonstrated more fibronectin accumulation in complement-sufficient rats than in C6-deficient rats (1.66 $\pm$ 0.28 versus 0.87 $\pm$ 0.13% area; $P < 0.05$) (Figure 6). The histologic and immunohistochemical results are summarized in Table 1.

Discussion

Proteinuric urine, which can be an independent mediator of progression rather than simply a marker of glomerular dysfunction, causes injury to the tubulointerstitium, leading to parenchymal damage and, ultimately, renal scarring and insufficiency (reviewed in references (14 through 18). Activation of complement in proteinuric urine can be one of the mechanisms by which proteinuria causes renal pathologic changes.

Although complement components are not filtered by glomeruli under normal conditions, they are present in the urine of patients with nephrotic syndrome and nonselective proteinuria (41,42) and can be activated directly by the brush border, leading to tubulointerstitial damage. A positive correlation between the occurrence of tubular C3 deposits and urinary complement excretion in patients with nonselective proteinuria supports this hypothesis (43). Proximal tubules are the principal sites of ammonium production (44). Free-base ammonia can disrupt the internal thiolester bond of the third component of complement, endowing it with “C3b-like” properties (45). C3 modified by ammonia (also termed amidated C3) forms the alternative pathway convertase and initiates cleavage of C3 and C5. Although ammonia may activate the complement cascade on the basolateral membrane of proximal tubular cells, preferential secretion of ammonia into the tubular lumen makes C3 amidation, with consequent activation of the alternative pathway, at the brush border more likely. Our immunofluorescence studies, showing complement component deposition at the brush border of the proximal tubules, demonstrated complement activation at the apical membrane, and previous studies support this notion. Camussi et al. (24,25) showed that the brush border of proximal tubules directly activated the alternative complement pathway. Micropuncture studies demonstrated that the induction of proximal tubule lesions by intraluminal perfusion with fresh serum was prevented by inactivation of the complement system (46). The deleterious role of complement components in proteinuric urine was further emphasized by recent studies in animal models. Matsuo and colleagues (47,48) demonstrated that rat glomerulonephri-
tis models were associated with less severe tubulointerstitial damage when the complement components were pharmacologically depleted or when activation of the complement pathway was blocked with a recombinant complement regulatory protein. We also demonstrated more severe tubular damage in proteinuric animals that had been treated with antisense oligodeoxynucleotides to reduce expression of the complement regulatory protein Crry in tubules (61). However, the mechanism of the complement effect, i.e., whether C5b-9 (the terminal membrane attack complex) or the generation of chemotactic factors during the process of activation of the complement cascade plays an essential role, remains to be determined.

Table 1. Summary of the histologic and immunohistochemical studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C-Sufficient Rats at Day 7</th>
<th>C6-Deficient Rats at Day 7</th>
<th>C-Sufficient Rats at Day 12</th>
<th>C6-Deficient Rats at Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (mg/d)</td>
<td>88.4 ± 8.20</td>
<td>98.0 ± 10.6</td>
<td>140 ± 16.6</td>
<td>124 ± 12.8</td>
</tr>
<tr>
<td>TI damage scoring</td>
<td>0.59 ± 0.12</td>
<td>0.33 ± 0.12</td>
<td>1.98 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.81 ± 0.21</td>
</tr>
<tr>
<td>Vimentin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteopontin (% area)</td>
<td>3.42 ± 0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.96 ± 0.32</td>
<td>13.7 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.98 ± 1.13</td>
</tr>
<tr>
<td>PCNA (+ cells/hpf)</td>
<td>0.40 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.08</td>
<td>1.05 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>Fibronectin (% area)</td>
<td>1.66 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.88 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

* C, complement; TI, tubulointerstitial; PCNA, proliferating cell nuclear antigen.

* P < 0.05 versus C6-deficient group.
for C3 and negative staining for C5b-9 in tubules of C6-deficient proteinuric animals.

To confirm that rats without C5b-9 formation sustained less tubulointerstitial damage, we used three different markers of tubular injury and regeneration, i.e., vimentin, osteopontin, and PCNA. Vimentin, a member of the family of intermediate filaments, is expressed in glomeruli and renal vasculature, but not in tubular cells, in normal rats (49). In contrast, alterations of damaged tubules, regardless of pathogenesis, include neo-expression of vimentin (29,49–53). Complement-sufficient puromycin-treated rats showed more tubular injury, documented as more vimentin-positive tubules, in our studies.

Osteopontin is a secreted, negatively charged glycoprotein with cell-adhesive and chemotactic properties in vitro (reviewed in reference (54). Tubular osteopontin expression has been shown to be elevated in a variety of animal models of tubulointerstitial renal disease (51,55–58) and may mediate macrophage adherence and fibrosis (62). Pichler et al. (57) demonstrated that osteopontin levels were closely correlated with the degree of tubulointerstitial injury in various animal models. Although vimentin is a late-phase marker of tubular injury, osteopontin levels are increased during the early phase of tubular damage (57). Osteopontin expression was significantly increased in tubules of normocomplementemic rats at both day 7 and day 12, compared with C6-deficient animals.

In contrast to vimentin and osteopontin, which serve as markers of tubular damage, increased tubular PCNA expression has been suggested to be a marker of tubular regeneration after injury (57). Normocomplementemic rats exhibited more PCNA-positive tubular cells at day 12 in our studies. The increases in these tubular injury markers were also associated with greater accumulation of extracellular matrix (fibronectin) in complement-sufficient animals.

C5b-9 formation can lead to tubular and interstitial injury through several different mechanisms. Human proximal tubular epithelial cells exposed to C5b-9 attack showed cytoskeletal alterations, with disruption of the actin network, resulting in cell lysis (59). In response to complement attack, human proximal tubular cells also generated a variety of proinflammatory mediators, which may contribute to tubulointerstitial damage (59,60). These events were dependent on C5b-9 formation on the tubular epithelial surface membrane. Recent studies of patients with various glomerular disorders demonstrated that patients with or without tubulointerstitial lesions showed dep-
position of a variety of complement components in the tubulo-interstitium. However, increases in C5b-9 deposition were observed only for those with tubulointerstitial damage (28). These previous reports are consistent with our results, emphasizing a role for C5b-9 in the pathogenesis of tubulointerstitial damage induced by proteinuric urine.

Although our studies demonstrated an important role for C5b-9, some tubulointerstitial changes, although less significant, developed in C6-deficient animals. These are likely mediated by non-complement-dependent mechanisms such as glomerular cytokine release, transferrin-induced oxidative mechanisms, or direct injury to tubules caused by exposure to high protein concentrations in the urine (14–19).

In summary, these studies confirm and extend prior observations on the role of complement in the tubulointerstitial damage that occurs in diseases with nonselective proteinuria. Our studies, for the first time, demonstrate that this complement effect is mediated by proteinuria-induced C5b-9, which is present on proximal tubular brush borders. These findings suggest that tubular C5b-9 attack induced by proteinuria contributes to the loss of nephron function by inducing tubulointerstitial damage and that prevention of C5b-9 formation in tubules might slow the deterioration of renal function.

Acknowledgments

Portions of this work were supported by research grants from the U.S. Public Health Service (DK34198 and DK07467) and from the Northwest Kidney Foundation.

References


42. Kjalman A, Avital A, Myers BD: Renal handling of the third (C3) and fourth (C4) components of the complement system in nephrotic syndrome. *Nephron* 16: 333–343, 1976


