The Role of Complement in the Pathogenesis of Tubulointerstitial Lesions in Rat Mesangial Proliferative Glomerulonephritis

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Abstract. Persistent proteinuria and tubulointerstitial lesions are important signs of progressive renal disease. The purpose of this study was to assess the role of complement in the development of tubulointerstitial lesions in rats with proteinuria due to primary glomerulonephritis. Mesangial proliferative glomerulonephritis was induced in mononephrectomized rats by intravenous injection of monoclonal antibody (mAb) 1-22-3 (Clin Exp Immunol 102: 181–185, 1995). As early as 24 h after the injection, proteinuria became evident, persisted throughout the observation period, and was associated with mesangial cell proliferation and tubulointerstitial lesions when examined at 7 and 14 d after mAb administration. Deposition of rat C3 and C5b-9 was observed at the luminal surface of proximal tubules and in cellular debris present in the tubular lumen (group 1). Rats injected with mAb 1-22-3 and depleted of complement by injections of cobra venom factor starting at day 3 developed glomerulonephritis and proteinuria comparable to rats of group I, but complement deposition in the tubules and the tubulointerstitial lesions were markedly reduced (group II). Rats in group III were injected with mAb and, from day 3, with soluble complement receptor type 1, which became detectable at the luminal surface of proximal tubules and in the urine. Deposition of C5b-9 in tubular cells was not detectable, and the severity of tubulointerstitial lesions was reduced compared with rats in group I. These results indicate that, in this model of primary mesangial proliferative glomerulonephritis with proteinuria, the development of tubulointerstitial lesions is associated with activation of serum complement at the level of tubular brush border, and tubulointerstitial lesions can be reduced by inhibition of complement activity. (J Am Soc Nephrol 8: 1363–1372, 1997)

In patients with glomerulonephritis, persistent proteinuria is indicative of progressive disease. Several studies have demonstrated that the prognosis is better correlated with the degree of tubulointerstitial damage than with glomerular pathology (1–3). One of the possible links between glomerular injury and the subsequent tubulointerstitial lesion is the presence of an abnormal amount of plasma proteins in the glomerular filtrate (4), including albumin (5–7), lipoproteins (7,8), and transferrin (9).

Complement has also been considered an important causal factor of tubulointerstitial injury. In 1974, Sato and Ullrich reported that isotonic reabsorption by rat kidney proximal tubule was drastically inhibited after short perfusion with fresh sera from rats or other species (10). Complement was involved in this phenomenon, although the precise mechanisms of its activation remained unknown (11). Other in vitro studies showed that the alternative pathway of complement can be activated by the brush border of proximal tubular cells (12,13), possibly because of the lack of membrane-associated complement regulators at the C3 level (14). In the study presented here, we test the hypothesis proposed by Williams and Coles (15) that complement plays a pivotal role in the development of tubulointerstitial lesions associated with glomerulonephritis and proteinuria.

The results show that when increased amounts of plasma proteins leak into the tubular lumen, complement is activated at the level of brush border with consequent tubulointerstitial damage, which can be decreased by inhibition of complement activation. The implications of this phenomenon for the pathogenesis and treatment of human renal diseases are discussed.

Materials and Methods

Animals

Female Wistar rats weighing 130 to 150 g were purchased from Chubu Kagaku Shizai Co., Ltd. (Nagoya, Japan). They were allowed free access to food and water. The experiments were performed according to the Animal Experimentation Guide of Nagoya University School of Medicine.

Reagents

mAb 1-22-3 against rat mesangial antigen was raised according to a method described before (16). The characteristics of mAb 1-22-3 are
described elsewhere (17). Soluble complement receptor type 1 (sCR1) (18) was supplied by Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and T Cell Sciences, Inc. (Needham, MA). Cobra venom factor (CVF) was routinely purified as described previously (19). mAb 2A1 against rat C5b-9 was kindly provided by Dr. W. G. Couser (University of Washington, Seattle, WA) (20). FITC-labeled mouse mAb against rat leukocyte common antigen (clone OX-1) was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Rabbit antibodies against sCR1 were raised by injecting 1 mg of sCR1 intradermally with Freund’s complete adjuvant every 2 wk. One week after the third immunization, rabbits were sacrificed and the sera were collected. These antisera were tested by Western blot analysis against sCR1; the human erythrocyte membrane formed a single band at 220 kD, whereas sCR1 was detected at 206 kD. This reflects that sCR1 does not have transmembrane and cytoplasmic domains. Anti-sCR1 antisera did not react with normal human serum, normal rat serum, or normal rat kidney sections. Thus, rabbit anti-sCR1 antisera were monospecific for human sCR1.

**Development of Mesangial Proliferative Glomerulonephritis**

The disease was induced according to a method described before (21). Briefly, 1 h after removal of the left kidneys under ether anesthesia, rats were injected intravenously with 0.5 mg of mAb 1-22-3 in 0.5 ml of saline. Proteinuria was first detected 24 h after mAb injection and persisted until the day of sacrifice. Immediately after the injection (day 0), there was development of mesangial changes associated with glomerular deposition of mouse IgG (mAb 1-22-3) and rat C3 and C5b-9. On day 2, the mesangial deposition of complement was markedly decreased and, from day 3 on, the deposition of mouse IgG and rat C3 was no longer detectable. After day 3, mesangial expansion consisted of proliferated mesangial cells, and increased mesangial matrix became evident. These findings are in agreement with the hypothesis that, after day 3, glomerular pathology and proteinuria are complement-independent. In preliminary experiments, serum complement was depleted by injections of CVF initiated at days 1, 3, and 5. Fifty units of CVF were given every other day. Using this procedure, the 50% complement hemolytic activity (CH50) level was kept below detection until the day of sacrifice (day 7). Urinary protein excretion was measured every day. Rats were sacrificed at day 7, and the renal pathology was assessed by light and immunofluorescence microscopy. Complement depletion starting at day 1 significantly reduced the glomerular lesions and proteinuria when compared with noncomplementemic animals. In contrast, complement depletion starting at day 3 or day 5 did not reduce glomerular pathology and proteinuria (data not shown). On the basis of these preliminary experiments, the relationship between complement and tubulointerstitial lesions was studied in rats injected every other day with CVF starting from day 3.

**Experimental Protocol**

Forty-two rats were divided into seven groups (Table 1). Rats of group I were injected with nephrotoxic mAb 1-22-3 as described above. These rats were used as the nephritic controls and sacrificed either at day 7 (group I-a) or day 14 (group I-b). Rats of group II-a were treated in the same way except for intravenous injection of 50 U of CVF at days 3 and 5. In rats of group II-b, injections of CVF were performed at days 3, 5, 7, 9, 11, and 13, and they were sacrificed at day 14. Rats of group III were treated in the same way as rats of group I, but also injected intraperitoneally with 20 mg/kg body wt of sCR1 every 8 h from day 3 to day 6. Rats of group IV-a and IV-b were mononephrectomized and injected intravenously with 0.5 ml of saline. Urinary protein excretion was measured before induction of nephritis, and at days 3, 5, 7, 11, and 14 by overnight urine collection (16 h) using metabolic cages. Rats of group I-a, II-a, III, and IV-a were sacrificed at day 7, and rats of group I-b, II-b, and IV-b were sacrificed at day 14. Renal tissue was processed for histological and immunohistological examination.

**Histology and Immunohistology**

For light microscopic examination, methacraln (methanol:chloroform:acetic acid = 6:3:1)-fixed (22) and paraffin-embedded kidney tissues were cut at 2 μm and stained with periodic acid-Schiff. Tubulointerstitial lesions were studied in the cortex and in the outer medulla with semiquantitative evaluation of tubular cast, tubular dilation, and tubular degeneration according to a method described previously (23). Under high magnification (×400), 20 randomly selected microscopic fields, which covered more than 70% of the cortex and more than 90% of the outer medulla, were examined. In each field, these changes were graded as follows: 0, normal; 1, <30%; 2, 30 to 70%; 3, >70%. In each rat, the average score of 20 fields was used as index of tubulointerstitial lesions.

To assess glomerular lesions, 40 glomeruli in each section were examined. There were three characteristic lesions in this model (i.e., mesangiolysis, expansion of mesangial area, and mesangial cell proliferation). Mesangiolysis, defined by the destruction of mesangial structure and the presence of aneurysmal dilation of glomerular capillaries, was assessed by the following formula:

**Mesangiolysis**

\[
\text{Mesangiolysis} = \frac{\text{Number of glomeruli with mesangiolysis}}{\text{Number of glomeruli examined (40)}} \times 100 \%
\]

**Table 1. Experimental protocol**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Operation</th>
<th>Reagent Injection</th>
<th>Treatment</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-a</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>mAb 1-22-3 (i.v.)</td>
<td>None</td>
<td>Day 7</td>
</tr>
<tr>
<td>I-b</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>mAb 1-22-3 (i.v.)</td>
<td>None</td>
<td>Day 14</td>
</tr>
<tr>
<td>II-a</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>mAb 1-22-3 (i.v.)</td>
<td>CVF/days 3</td>
<td>Day 7</td>
</tr>
<tr>
<td>II-b</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>mAb 1-22-3 (i.v.)</td>
<td>CVF/days 3, 5, 7, 9, 11, and 13 (i.v.)</td>
<td>Day 14</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>mAb 1-22-3 (i.v.)</td>
<td>CR1/days 3 through 6 (i.p.)</td>
<td>Day 7</td>
</tr>
<tr>
<td>IV-a</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>Saline (i.v.)</td>
<td>None</td>
<td>Day 7</td>
</tr>
<tr>
<td>IV-b</td>
<td>6</td>
<td>Uninephrectomy</td>
<td>Saline (i.v.)</td>
<td>None</td>
<td>Day 14</td>
</tr>
</tbody>
</table>

* mAb, monoclonal antibody; i.v., intravenously; CVF, cobra venom factor; CR1, recombinant soluble human complement receptor 1; i.p., intraperitoneally.
The mesangial expansion was graded as follows: 0, no expansion; 1, mesangial expansion accounting for less than 25% of glomerular area (not including Bowman's space); 2, 25 to 50%; 3, 50 to 75%; 4, more than 75% (22). In each rat, the average score of 40 glomeruli was used as an index of glomerular injury.

For immunofluorescence microscopy, kidney tissues were snap frozen, cut at 2 μm by a cryostat, and fixed in acetone. Sections were stained by FITC-labeled goat antibodies against rat C3 (Cappel Laboratories, West Chester, PA), mAb 2A1 against rat C5b-9, followed by FITC-labeled rabbit antibodies against mouse IgG (Cappel). For the detection of sCR1, frozen sections were first incubated with rabbit anti-sCR1 (×200) for 15 min at room temperature and then stained with FITC-labeled goat anti-rabbit IgG. For the analysis of glomerular and interstitial leukocyte infiltration, sections were stained by FITC-labeled OX1. After washing with phosphate-buffered saline, all of the sections were covered with 90% glycerol containing p-phenylenediamine (24) and examined by two observers using an epifluorescence microscope (Olympus Optical Co., Tokyo, Japan). Deposition of rat C3 and C5b-9 was assessed differentially in cell debris, brush border, and tubular basement membrane (TBM). The staining was semiquantitatively graded from 0 to 3 according to the criteria described previously (23). The number of leukocytes was counted in 20 randomly selected glomeruli or 20 microscopic fields under high magnification (×400). In each rat, the average number was considered representative of cellular infiltration.

**Serum Complement Activity**

Blood samples were drawn from the tail vein into syringes containing ethylenediamine tetra-acetic acid (Sigma Chemical Co., St. Louis, MO) before surgery and at days 3, 5, 7, and 14. In rats of groups II and III, blood samples were collected before injection of CVF or sCR1. Serum CH$_{50}$ level was measured using sensitized sheep red blood cells (Ishizu Pharmaceutical Co., Osaka, Japan) according to the manufacturer's instructions. Serum complement activity (percentage) was calculated using the following formula:

\[
\text{Serum Complement Activity} = \frac{\text{CH}_{50} \text{ at the day of interest}}{\text{CH}_{50} \text{ before surgery}} \times 100 \ (%)
\]

**Urinary Protein**

Rats were housed in metabolic cages overnight (16 h) on days 0, 3, 5, 7, 11, and 14. Total protein excretion into urine was measured by pyrogallol red method (25). The urinary albumin was measured by enzyme-linked immunosorbent assay, using NEPHRAT (Exocell Inc., Philadelphia, PA).

**Detection of Urinary sCR1**

Rat urine was analyzed by Western blot to identify the presence of sCR1. Urine samples were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoresed proteins were then transferred to the nitrocellulose membrane and washed. The membrane was then incubated with rabbit anti-sCR1 followed by peroxidase-labeled goat anti-rabbit IgG at room temperature. The reaction was visualized by incubating the membrane in diaminobenzidine solution and hydrogen peroxide (26).

**Statistical Analyses**

All values were expressed as mean ± SEM. Statistical analysis was performed by one-factor ANOVA. When a significant difference was present, analysis was further performed using Scheffe's F test between two groups. A P value less than 0.05 (5%) was considered significant.
Results

Urinary Protein

The levels of proteinuria are shown in Figure 1A. In rats of groups I, II, and III, protein excretion started to increase within 24 h after injection of mAb 1-22-3 and persisted until the day of sacrifice. The degrees of proteinuria in rats of groups I, II, and III were comparable throughout the experiments. Rats of group IV did not develop proteinuria. The measurement of urinary albumin excretion revealed similar results (Figure 1B).

Serum CH$_{50}$

CH$_{50}$ levels were reduced to less than 5% of baseline value in rats of group II, and to 18% in group III at day 5 and afterward. CH$_{50}$ levels were normal in rats of groups I and IV throughout the study (Figure 2).

Figure 3. Light microscopy pictures of representative glomeruli from rats in groups I (A), II (B), and III (C). Each glomerulus shows significant expansion of the mesangial area due to increased mesangial cells and matrix. In the cortex of rats in group I (A), there is significant dilatation and degeneration of proximal tubules and mild cellular infiltration (arrows) in the interstitium. In kidneys of rats of groups II (B) and III (C), the tubular changes are less severe or absent. Magnification (Panels A through C), ×400.

Figure 4. Quantitative and semiquantitative data of glomerular injury. (A) Total glomerular cells at day 7 and day 14 assessed by number of nuclei per glomerular cross-section. There is a significant increase in groups I, II, and III compared with group IV. There is no significant difference among groups I, II, and III. (B) Mesangiolysis. In rats of groups I-a, II-a, and III, approximately 20% of glomeruli have mesangiolysis at day 7. At day 14, approximately 10% of glomeruli have mesangiolysis in groups I-b and II-b. There is no significant difference among these groups. Glomeruli from group IV rats do not show mesangiolysis. (C) Semiquantitative assessment of mesangial expansion. There is significant mesangial expansion in glomeruli of rats from groups I, II, and III. The degree of mesangial expansion is not significantly different among these three groups. Glomeruli from rats of group IV did not show mesangial expansion at day 7 or day 14.
Complement and Tubulointerstitial Injury

Figure 5. Light microscopy pictures of tubulointerstitium from outer medulla at day 7. (A) Group I; (B) group II; (C) group III. Note that tubular dilation, degeneration (flattening of tubular cells and loss of brush border) are evident in group I. There is also cellular infiltration in the interstitium. In groups II and III, these changes are less severe. Magnification (Panels A through C), ×400.

Light Microscopy Findings

Glomerular Lesions. Significant glomerular changes were observed in rats of groups I, II, and III at days 7 and 14. There were significant increases in glomerular cell number and marked expansion of mesangial area (Figure 3). Degrees of these changes did not significantly differ in these groups (Figure 4). Mesangiolysis was seen in approximately 20% of glomeruli of rats of group I-a at day 7 and in approximately 10% at day 14, which was not significantly different from that of group II-a or group III (Figure 4). The glomeruli of rats in group IV were normal.

Tubulointerstitial Lesions. Tubulointerstitial changes were observed in rats of group I. Tubular hyaline casts, dilation of tubules, vacuolar degeneration, numerous mitotic figures, loss of brush border, and detachment of tubular epithelial cells from TBM were observed both at day 7 (Figure 5) and day 14. Leukocyte infiltration was also seen in the tubulointerstitial area. Semiquantitative analysis revealed that the extent of tubular dilation and degeneration was significantly less in groups II and III than in group I, both in the cortex and in the outer medulla. The

Figure 6. Semiquantitative analysis of tubular lesions. (A) Cortex. (B) Outer medulla. Tubular degeneration and dilation are most severe in rats of groups I-a and I-b, less severe in rats of groups II and III, and absent in rats of groups IV-a and IV-b. There was no significant difference in hyaline casts among groups I, II, and III.
number of casts was not significantly different among groups I, II, and III in the cortex and in the outer medulla. In group IV rats, tubulointerstitial lesions were absent (Figure 6).

**Immunohistological Findings**

**Glomeruli.** There was no deposition of mouse IgG, rat IgG, and C3 in all rats at days 7 and 14. In rats of groups I, II, and III, there was a trace amount of C5b-9 in the mesangial area both at day 7 and day 14. This might represent a residue of C5b-9 inserted into the plasma membrane of mesangial cells before day 3. Leukocyte infiltration was significantly increased, in comparable proportion, in groups I, II, and III both at day 7 and day 14 (Figure 7A). The glomeruli of group IV rats were normal.

**Tubulointerstitium.** In rats of group IV, interrupted and linear deposition of C3 and C5b-9 was observed only in the TBM of proximal tubules. This staining pattern is also seen in the normal kidney (27), but the reasons are unknown. In rats of group I, deposition of C3 and C5b-9 was detected at the luminal surface of proximal tubules, in cellular debris, and in absorption droplets. The staining for C5b-9 was weaker than that for C3. C3 was almost undetectable in rats of group II, whereas it was weakly positive at the luminal side of proximal tubules in rats of group III. C5b-9 was almost negative in the brush border and cellular debris of rats of groups II and III (Figure 8). The results of semiquantitative analysis of complement deposition in the tubules are given in Figures 9 and 10. Leukocyte infiltration in the cortex and outer medulla was prominent in group I, mild in groups II and III, and absent in group IV (Figure 7B).

**sCR1 in Kidney and Urine**

In rats of group III, sCR1 was observed along the luminal surface of proximal tubular cells. By Western blot analysis, the urinary samples from group III, but not from groups I or II, showed a positive band at 206 kD (Figure 11).

**Discussion**

The results of the present study show that in rats with proteinuria of glomerular origin, the activation of complement at the luminal surface of the proximal tubular cells contributes to development of tubulointerstitial lesions. Interference with complement activation, either by systemic complement depletion or by local inhibition of C3 convertase, can significantly decrease the tubulointerstitial lesions without modifications of glomerular pathology. These findings provide new evidence for a role of complement in the development of tubulointerstitial injury associated with proteinuria.

In proteinuric conditions, several mechanisms could contribute to the activation of complement at the level of tubular brush border. First, the brush border of proximal tubules expresses large amounts of angiotensin-converting enzyme (28), and purified angiotensin-converting enzyme is reported to activate C1 (29). Second, the alternative pathway can be activated by the cleavage of the internal thiolester bond of C3 molecule by water (30) or ammonia (31). Thus, complement can be spontaneously activated via classical and alternative pathways in the luminal surface of proximal tubular cells. Once activated, C3 convertases can amplify the further cleavage of C3 molecules. This step is usually suppressed by the fluid phase and cell membrane-bound complement regulators (32). Therefore, at this site, complement activation may occur when plasma proteins, including complement components, reach the tubular lumen.

As for the mechanisms of complement-mediated tubulointerstitial injury in the present model, direct and indirect effects of complement activation are considered. Concerning the direct effects, C5b-9, the final product of the complement cascade, is the most probable effector of tubulointerstitial damage. C5b-9 was formed on proximal tubular cells in rats of group I. These cells could be either detached from TBM or activated to produce various kinds of biologically active substances, as in leukocytes (33) or cultured mesangial cells (34) binding non-lethal amounts of C5b-9. Moreover, in normal human proximal tubular cells in culture, the insertion of C5b-9, but not the binding of C3, on the plasma membrane induces production of reactive oxygen species (13). In humans, C5b-9 can be detected in the urine of patients with nonimmunological glomerular disease such as focal glomerulosclerosis and diabetic

![A Glomerulus](image)

**Figure 7.** Leukocyte infiltration in glomeruli (A) and interstitium (B). There is significant and comparable glomerular infiltration of leukocytes in groups I, II, and III. However, leukocyte infiltration in the tubulointerstitial tissue was most evident in group I rats.
nephropathy, indicating that complement activation is occurring in the tubular lumen (35). In contrast, it is also probable that leukocytes, infiltrated into renal interstitium in a complement-dependent manner, injured the tubulointerstitial tissue. These mechanisms are not mutually exclusive.

The prophylactic or therapeutic role of sCR1 has been
shown in various models of experimental inflammation, including reversed passive Arthus reaction (36), postischemic heart injury (18,37), and antibody-mediated glomerulonephritis (38). In our rats injected with sCR1 (group III), the serum level of CH₅₀ was reduced by 80% and sCR1 was detected in the urine. Furthermore, sCR1 was shown at the luminal surface of the proximal tubular cells. The deposition of C5b-9 was significantly decreased compared with control rats of group I. Concomitantly, the severity of tubulointerstitial lesions was significantly diminished and was comparable to that of rats decomplemented by CVF (group II). These results suggest that sCR1 filtered into tubular lumen inhibited complement activation at the surface of proximal tubular cells. Tubulointerstitial damage is the final common pathologic pathway of many glomerular diseases. Our results show that persistent proteinuria can induce complement-dependent tubulointerstitial lesions. The strategy to prevent or retard the progression of tubulointerstitial lesions in man should be focused first on the reduction of proteinuria. When this goal cannot be or is only incompletely achieved, the inhibition of complement activation at the level of tubular brush border may help prevent or minimize the development of tubulointerstitial damage. For instance, administration of sCR1, a relatively safe regulator of complement activation, may be considered.

**Figure 9.** Semiquantitative analysis of C3 deposition in cortex (A) and outer medulla (B).

**Figure 10.** Semiquantitative analysis of C5b-9 deposition in cortex (A) and outer medulla (B).

**Figure 11.** Western blot analysis of urinary sCR1. Lanes A and B, human erythrocyte membrane (positive control). Lanes C and D, sCR1 (positive control). Lanes E and F, Urine samples from rats of group III at day 7. Lane G, Urine sample from a rat of group I at day 7. Lane H, Urine sample from a rat of group II at day 7. Note that only samples from group III show a 206-kD band.

**Acknowledgments**

The authors thank Dr. W. G. Couser for providing mAb against rat C5b-9 neoantigen. Part of this work was supported by the 1996 Research Grant from the Aichi Kidney Foundation and a grant from
References


33. Morgan BP, Campbell AK: The recovery of human polymorphonuclear leukocytes from sublytic complement attack is mediated by changes in intracellular free calcium. *Biochem J* 231: 205–208, 1985


