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Role of Complement in Acute Tubulointerstitial Injury of Rats with Aminonucleoside Nephrosis

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The present work was designed to elucidate the *in vivo* role of complement in the proteinuria-associated tubulointerstitial injury. Rats were intravenously injected with puromycin aminonucleoside, and massive proteinuria was observed within 5 days. Prominent tubulointerstitial injury characterized by proximal tubular degeneration, tubular dilatation, and leukocyte infiltration were observed 7 days after injection. C3 and C5b-9 were observed in the luminal side of proximal tubular cells. Renal function, assessed by inulin and para-aminohippurate clearance, was significantly decreased. To assess the role of complement in this model, rats were injected with either cobra venom factor or soluble recombinant human complement receptor type 1 starting at day 3. These manipulations significantly improved tubulointerstitial pathology and para-aminohippurate clearance without affecting the degree of proteinuria. Deposition of C3 and C5b-9 was not detected in the kidney of rats depleted of complement by cobra venom factor. In rats treated with soluble complement receptor, C3 was still detected in the tubules, but deposition of C5b-9 was not observed. Soluble complement receptor was detected at the site of C3 deposition and in the urine. These data strongly suggest that complement plays a pivotal role in proteinuria-associated tubulointerstitial injury and that systemic complement depletion or inhibition of complement in the tubular lumen may diminish the tubulointerstitial damage. *(Am J Pathol 1997, 151:539–547)*

In many glomerular diseases, the degree of persistent proteinuria is a prognostic indicator of renal function.[1] In the setting of persistent proteinuria, significant amounts of plasma proteins are filtered into the tubular lumen. The exposure of plasma proteins to the proximal tubular cells may either damage or modulate the function of these cells resulting in the subsequent tubulointerstitial injury.[2–4] The importance of tubulointerstitial injury has been proposed from studies of human biopsies that show that renal functional prognosis is better correlated with the degree of tubulointerstitial damage than with the glomerular injury.[5] Because of this, many efforts are now focused on the investigation of the mechanisms of proteinuria-induced tubulointerstitial injury. Several plasma proteins, including albumin,[6] lipoproteins,[7] and transferrin,[8,9] all of which are usually absent in the tubular lumen in the normal condition, are thought to have direct and indirect toxicities for the tubules. Endothelin 1,[7] osteopontin,[10] macrophage chemoattractant protein 1 (MCP-1),[11] a novel lipid chemoattractant,[6] insulin-like growth factor,[12] and reactive oxygen metabolites[13] are subsequently generated in the injured or activated proximal tubular cells, and all of these are thought to contribute to the development of proteinuria-associated tubulointerstitial injury.

In addition to these substances, complement components filtered across the glomerular barrier into the tubular lumen are also postulated to have important roles in the development of proteinuria-associated tubulointerstitial injury. It was demonstrated by the *in vitro* studies using normal human kidney sections that the alternative pathway of complement was activated on the brush border of the proximal tubular cells.[14] This might be due to the lack of membrane regulators of complement at C3 level in the proximal tubule brush border.[15] In fact, Eddy has described the deposition of C3 in the luminal surface of proximal tubules of rats with protein overload nephropathy.[3] Although these data support the importance of complement, direct evidence is further required for the establishment of the role of complement in proteinuria-associated tubulointerstitial injury.

The present work was designed to obtain the direct evidence for the pivotal role of complement in the proteinuria-associated tubulointerstitial injury *in vivo*. Using the puromycin aminonucleoside (PAN) nephrosis model, it was clearly demonstrated for the first time that tubulointerstitial injury associated with proteinuria was at least partially mediated by complement that was filtered through the glomerular barrier and activated on the proximal tubule cells. Furthermore, inhibition of complement

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activation in the tubular lumen by the soluble recombinant human complement receptor type 1 (sCR1)[16,17] significantly reduced the histological change of tubulointerstitium and lessened impairment of renal function induced by the heavy proteinuria. These data may have relevance for the better understanding of tubulointerstitial damage mediated by proteinuria.

Materials and Methods

Animals

Female Wistar rats weighing approximately 150 g were purchased from Chubu Kagaku Shizai Co. (Nagoya, Japan) and were allowed free access to food and water. The experiment described here was carried out according to The Animal Experimentation Guide of Nagoya University School of Medicine.

Reagents

PAN was purchased from Sigma Chemical Co. (St. Louis, MO). Cobra venom factor (CVF) was routinely purified as described before.[18] Characteristics of sCR1 were described previously[16] and was kindly provided by Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and T Cell Sciences (Needham, MA). Mouse monoclonal antibody 2A1[19] directed against rat C5b-9 was kindly provided by Dr. W. G. Couser (University of Washington, Seattle, WA). Antiserum against CR1 was obtained by immunization of sCR1 to the rabbits. Rabbit anti-sCR1 antiserum was tested by Western blot analysis using normal human erythrocyte membrane and normal human serum, and it was proved to be monospecific against human CR1.

Serum Complement Activity

Blood samples were drawn from the tail vein into syringes containing EDTA (Sigma) at days 0, 3, 5, and 7. Serum was kept frozen at −70°C until measurement. The serum level of 50% complement hemolytic activity (CH50) was determined using sensitized sheep red blood cells (Ishizu Pharmaceutical Co., Osaka, Japan) according to the manufacturer's direction. Excess amount of Ca^{2+} was added to the serum at the time of measurement.

Urinary Protein Excretion

Rats were housed in metabolic cages overnight (16 hours) at days 0, 3, 5, and 7. Urinary protein was measured by a pyrogallol red method.[20] Urinary albumin was measured by enzyme-linked immunosorbent assay using a rat albumin measuring kit (NEPHRAT, Exocell, Philadelphia, PA).

Urinary Excretion of sCR1

sCR1 in the urine samples was detected by the Western blot analysis according to a method described before.[21] Urine samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with skimmed milk, membrane was incubated with polyclonal rabbit anti-sCR1 and then peroxidase-labeled goat anti-rabbit IgG (Cappel, West Chester, PA). The reaction was visualized using diaminobenzidine and hydrogen peroxide.

Table 1. Experimental Protocol

Group	n	PAN	Reagent
I	8	150 mg/kg (i.v.)	None
II	7	150 mg/kg (i.v.)	CVF 50 U/body (i.v.) at days 3 and 5
III	7	150 mg/kg (i.v.)	sCR1 20 mg/kg (i.p.)/ 8 hours at days 3–7
IV	6	Saline	None

PAN, puromycin aminonucleoside; CVF, cobra venom factor; sCR1, recombinant soluble complement receptor type 1.

Experimental Protocol

Twenty-two rats were intravenously injected with 150 mg/kg PAN at day 0. Eight rats were not treated with any reagent after PAN injection (group I). Seven rats were depleted of complement starting at day 3 and afterwards by intravenous injection of 50 U of CVF at days 3 and 5 (group II). Another seven rats were intraperitoneally injected with 20 mg/kg sCR1 every 8 hours starting at day 3 (group III). Six rats were injected with 0.5 ml of saline at day 0 (group IV) and studied as normal controls for histopathological examinations. All rats were sacrificed at day 7. The experimental protocol is summarized in Table 1.

Histology and Immunohistology

At the time of sacrifice, the kidneys were processed for the studies by light and immunofluorescence microscopy. For light microscopy, part of the kidney tissue was fixed in methyl Carnoy's fixative overnight and was embedded in paraffin. Two-micron-thick sections were stained with periodic acid-Schiff reagents. Quantitation of tubulointerstitial injury was performed according to a method described before.[22] The kidney section was arbitrarily divided into four regions, ie, cortex, outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla. Using semiquantitative indices, sections were analyzed for the evaluation of acute tubulointerstitial damage. At each region of each rat, extents of tubular cast formation, tubular dilatation, and tubular degeneration (vacuolar change, loss of brush border, detachment of tubular epithelial cells, and condensation of tubular nuclei) were scored according to the following criteria: 0, normal; 1, less than 30%; 2, 30 to 70%; 3, more than 70% of the pertinent area.

For immunofluorescence microscopy, part of the kidney tissue was snap frozen in liquid nitrogen and kept frozen at −70°C until use. Two-micron-thick sections

were cut by a cryostat and fixed in acetone. Sections were then stained by fluorescein isothiocyanate (FITC)-labeled goat antibody against rat C3 (Cappel) and rabbit anti-rat IgG (Cappel). For the detection of C5b-9, sections were incubated with mouse monoclonal antibody 2A1 and then FITC-labeled goat antibody against mouse IgG (Cappel). Localization of rat albumin was examined to assess the nonspecific uptake of filtered plasma protein by proximal tubular cells. Sections were first reacted with rabbit anti-rat albumin (Cappel) followed by the incubation with FITC-labeled goat anti-rabbit IgG (Cappel) absorbed by normal rat serum. Deposition of rat C3 and C5b-9 in the kidney was semiquantitatively scored according to the extent of deposition in each region using the following criteria: 0, normal; 1, less than 20%; 2, 20 to 40%; 3, 40 to 60%; 4, 60 to 80%; 5, more than 80% of the pertinent area. To assess complement activation on the surface of tubular cells, the staining was counted as positive only when the deposition of C3 or C5b-9 was seen on the apical site of tubular cells. To detect the localization of sCR1, tissue sections were stained with rabbit anti-sCR1 and FITC-labeled goat anti-rabbit IgG (Cappel). For analysis of interstitial macrophage infiltration, sections were stained by mouse monoclonal antibody against rat macrophage/monocyte (clone ED1; BMA Biochemicals AG, Augst, Switzerland) and FITC-labeled goat anti-mouse IgG. The number of positive cells was counted in 10 randomly selected microscopic fields of each region under higher magnification (×400). After washing with PBS, all of the sections were covered with 90% glycerol containing *p*-phenylenediamine[23] and were examined by two blinded observers using an epifluorescence microscope (Olympus Optical Co., Tokyo, Japan).

For electron microscopy, tissue fragments were fixed in 2.5% glutaraldehyde in 0.1 mol/L PBS for 2 hours. They were then processed by 2% OsO_4 for 2 hours, dehydrated, and embedded in Epon 812. Ultrathin sections were double stained with uranium and lead and were observed by a JEM100CX electron microscope (Tokyo, Japan).

Renal Function Study

In a separate set of experiment, studies for renal function were performed. Rats of groups I, II, and III were treated in the same way as described above. Rats of group IV were age- and sex-matched controls. Six rats in each group were examined. At day 7, polyethylene tubes were cannulated into the right jugular vein, left internal carotid artery, and bladder under pentobarbital anesthesia. To determine inulin and para-aminohippurate (PAH) clearance, a solution of saline containing inulin (6 mg/ml) and PAH (1 mg/ml) was infused from the venous catheter after a priming dose of 100 mg/kg body weight inulin. After a 30-minute period of equilibration, urine and blood samples were collected from the bladder and carotid artery. The plasma and urine concentration of inulin and PAH was measured by high-performance liquid chromatography.[24]

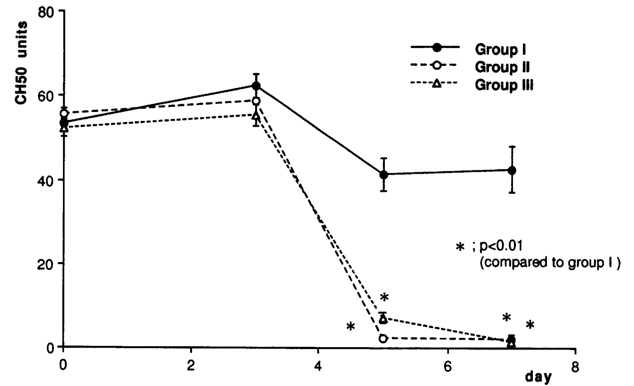

Figure 1. Serum complement activity was determined by 50% complement hemolytic activity (CH50). One CH?50 unit is defined by the complement hemolytic activity in 1 ml of serum that causes hemolysis of 50% of 5×10^8 properly sensitized sheep blood cells in Veronal-buffered saline (pH 7.4 containing Mg^{2+} and Ca^{2+}; total volume, 7.5 ml) at 37°C for 60 minutes. In group I rats, there was no significant change of CH50 level. In groups II and III, CH50 was nearly 0 after day 5. *Significant difference of group I *versus* groups II and III. There was no significant difference between groups II and III.

Statistical Analysis

All values are provided as mean ± standard error. Statistical analysis was performed by one-factor analysis of variance. When a significant difference was present, statistical analysis was further performed using Scheffe's *F*-test between two groups. A significant difference was set when the *P* value was less than 0.05 (5%).

Results

Serum Complement Activity

In group I rats, serum CH50 did not change significantly throughout the experiment. In groups II and III, serum CH50 decreased significantly at days 5 and 7 (Figure 1).

Urinary Protein Excretion

In rats of groups I, II, and III, urinary protein was not increased until day 3 and significantly increased at day 5 and afterwards. The total amount of urinary protein excretion was not significantly different among these three groups. Similarly, urinary albumin excretion was not significantly different among these groups (Figure 2). Urinary protein and albumin excretion in normal rats (group IV) was always less than 2 mg/16 hours (data not included in Figure 2).

Urinary Excretion of sCR1

In group III rats, urine sCR1 was detected by Western blot analysis at days 5 and 7. In rats of groups I and II, sCR1 was not detected in the urine (Figure 3).

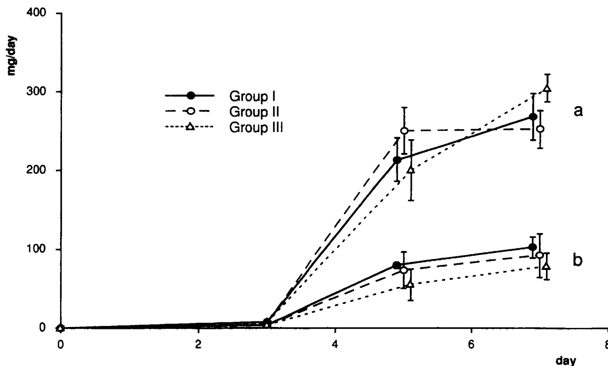

Figure 2. The effect of sCR1 on urinary excretion of total protein (**a**) and albumin (**b**). Urinary excretion of protein and albumin was not increased before day 3 and was remarkably increased at day 5. The degree of proteinuria and albuminuria was not significantly different among the three groups.

Histological Findings

Light Microscopy

In group I, dilatation of tubular lumina was prominent, especially in the cortex and outer stripe of outer medulla. Tubular cast formation and tubular epithelial cell degeneration as assessed by the loss of brush border and flattening and detachment of epithelial cells were also prominent. In contrast, tubular dilatation and degeneration were significantly suppressed in groups II and III. Tubular cast formation was not significantly different among these three groups (Figure 4). These results are semiquantitatively summarized in Figure 5. In rats of group IV (control or normal rats), there was no abnormal findings in the tubulointerstitial tissue (score = 0, data not included in Figure 4). In contrast to the tubulointerstitium, glomeruli showed a normal appearance in all rats (data not shown).

Electron Microscopy

In group I rats, partial loss of brush border, degeneration of organelles, detachment of tubular cells from tubular basement membrane, presence of electron-dense particles (absorption droplets), and infiltration of mononuclear cells into the tubular lumen were observed in cortex and outer medulla. In rats of groups II and III, those changes were much less prominent and were almost comparable to the normal subjects (group IV) except for absorption droplets. As for the glomeruli, changes in the podocytes such as diffuse fusion of foot processes, vacuolar formation, and condensation of electron-dense fibrils along glomerular basement membrane were observed in rats of groups I, II, and III. The degree of these changes were comparable among these three groups.

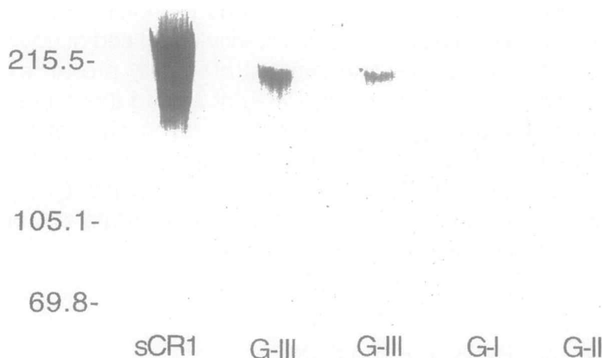

Figure 3. Western blot analysis for urine sCR1. **Lane 1**, positive control (sCR1); **lanes 2** and **3**, urine samples from group III (G-III) rats; **lane 4**, urine sample from a group I (G-I) rat; **lane 5**, urine sample from a group II (G-II) rat. Note that sCR1 was detected only in the urine of group III rats at 206 kd. sCR1 was not detected in the urine of groups I and II rats.

Deposition of Complement Components

In group I, prominent deposition of C3 and C5b-9 was seen in the apical side of the proximal tubules. Deposition of C3 and C5b-9 was scarcely seen in distal or collecting tubules. In group II rats, deposition of C3 and C5b-9 was hardly detectable in the apical side of proximal tubular cells or in the tubular lumen. In group III rats, C3 deposition was seen in the luminal aspect of proximal tubules, but the degree of C3 deposition was significantly milder in group III than in group I. Deposition of C5b-9 in the tubules of group III rats was much less prominent than that of C3. These data are shown in Figures 6 and 7. In rats of group IV (normal controls), there was no abnormal deposition of complement components (score = 0). Rat albumin was detected only very weakly in the lumen and luminal surface of occasional proximal tubules. In a very limited number of proximal tubules, rat albumin was strongly detected in the intracellular absorption droplets. The distribution and degree of albumin staining was not different among rats of groups I, II, and III. Albumin was not stained in control (group IV rats). Rat IgG was not stained in all groups. Glomerular deposition of complement components and rat IgG was undetectable in all rats.

Localization of sCR1

In groups I, II, and IV rats, there was no staining for sCR1 in the kidney. In group III, prominent staining of sCR1 in the glomerulus and vasculature was seen. Significant binding of sCR1 was also observed in the apical site of proximal tubules.

Macrophage Infiltration

According to the previous studies, it has been agreed that infiltrating cells in acute PAN nephrosis mainly consisted of macrophages.[2] In group I rats, there were large numbers of infiltrating macrophages in the entire interstitial area when compared with group IV rats. In contrast, macrophage infiltration was significantly suppressed in all layers of the kidney in group II and III rats (Figure 8).

Renal Function Study

In group I rats, marked decrease of both inulin and PAH clearance was noted compared with group IV (normal)

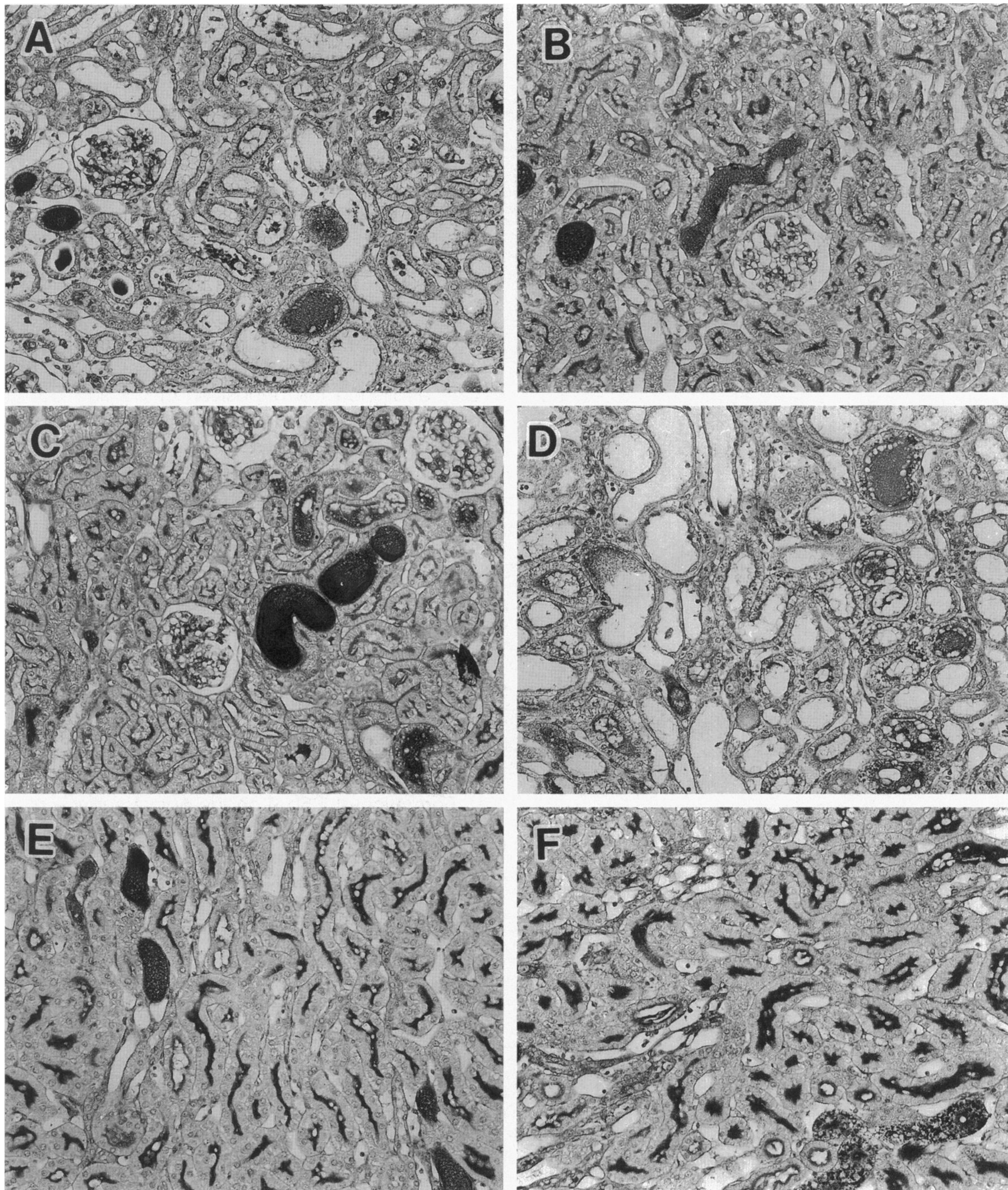

Figure 4. Light microscopic pictures of renal cortex (**A** to **C**) and outer stripe of outer medulla (**D** to **F**). Magnification, ×400. **A** and **D**: Group I. **B** and **E**: Group II. **C** and **F**: Group III. Tubular dilatation, degeneration, and interstitial mononuclear cell infiltration was significantly observed in group I. In contrast, these changes were significantly less prominent in groups II and III.

rats. PAH clearance was significantly improved to the normal level in group II and III rats. In contrast, inulin clearance was not significantly improved in group II and III rats and still remained far below the normal level. These data are summarized in Figure 9.

Discussion

In normal kidneys, small amounts of complement component C3 are always activated by the cleavage of its internal thiolester bond by the reaction with the water molecule[25] or ammonia.[26] The activated C3 can bind to factor B, which is cleaved by factor D to form C3 convertase. Although this C3 convertase has the ability to cleave the C3 molecule into C3a and C3b, further reaction is usually inhibited by the complement regulatory proteins present on the cell membrane or in plasma.[27] The importance of membrane-type complement regulators was demonstrated in our previous work,[18] which suggested that the inhibition of membrane-type complement regu-

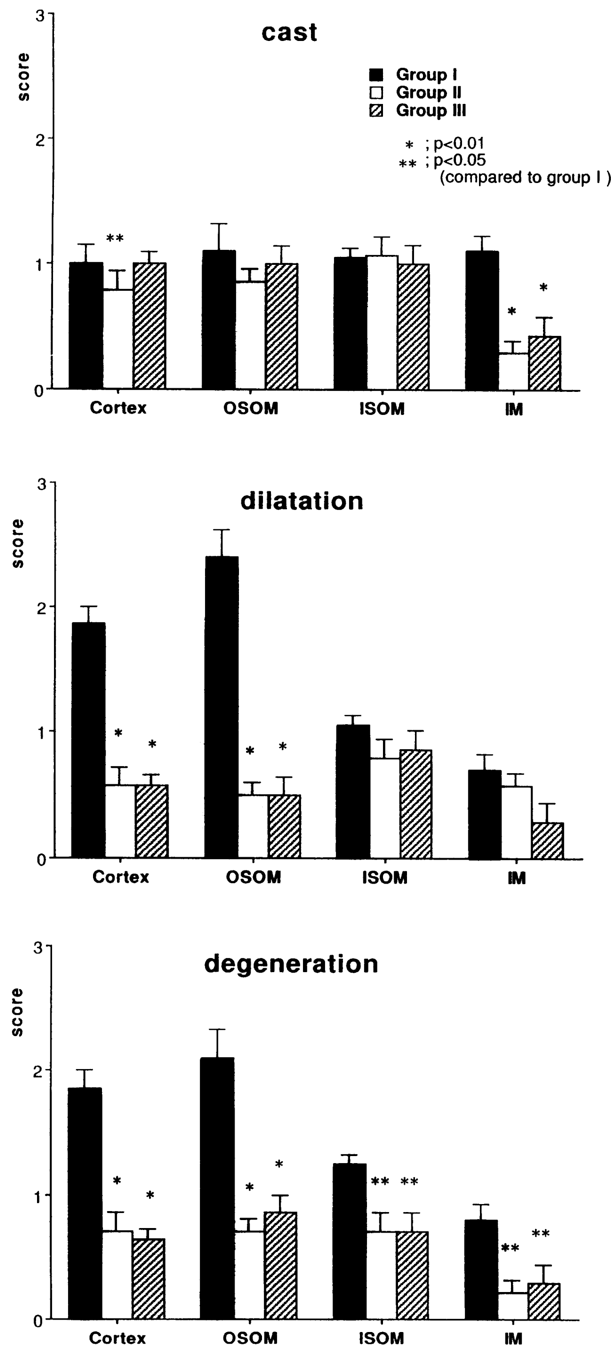

Figure 5. Quantitative measurement of tubular injury. In group I rats, tubular dilatation and tubular cell degeneration were more prominent than in groups II and III rats. In contrast, tubular cast formation was not significantly different among these three groups. **Asterisks** indicate the significant difference of group I *versus* groups II and III. There was no significant difference between groups II and III.

lator *per se* could induce complement activation on the cell surface regardless of the presence of fluid-phase complement regulators. Interestingly, another observation by our group demonstrated that membrane-bound complement regulatory proteins active at the level of C3 convertase are absent from the luminal surface of the proximal tubular cells of the normal human.[15] Thus, there is good reason to postulate that complement components, which are usually absent in the lumen of the proximal tubules, may be activated on the apical surface of proximal tubular cells in the proteinuric condition. This notion was affirmed *in vivo* in the present work by the following observations. First, not only C3 but also C5b-9 was deposited in the apical side of proximal tubular cells in group I rats at day 7. There was no deposition of C5b-9 in the glomeruli, and serum complement activity (CH50) was not significantly reduced. Schulze and his co-workers reported that C5b-9, when intravenously administered as zymozan-activated serum, was undetectable in the urine of PAN nephrosis rats.[19] These facts strongly suggested that deposition of C5b-9 in the tubules was the consequence of activation of the terminal complement pathway *in situ*. Second, serum complement depletion by CVF abolished the deposition of C3 and C5b-9 in the proximal tubules despite the finding that the degree of urinary protein excretion was not significantly altered. This fact clearly indicated that the deposition of C3 and C5b-9 was dependent on the serum complement activity.

At the present time, it is not clear whether proximal tubular cells can produce all kinds of the complement proteins or not as reported in the astrocytes.[28] But, there have been reports demonstrating that renal tubular epithelial cells can at least express the genes for complement components C3 and C4.[29,30] Therefore, the possibility that locally produced complement proteins are another source of urinary complement and contribute to the tubular injury is not excluded. In this sense, the regulatory mechanisms of complement production in the tubular cells must be further investigated.

Formation of C5b-9 on the apical surface of proximal tubular cells is thought to be the main event that modulates or injures the function of the proximal tubular cells. Biancone has shown using cultured human proximal tubular cells that these cells can activate complement via the alternative pathway and that they undergo subsequent cytoskeletal alterations. In addition, production by these cells of superoxide anion and hydrogen peroxide and the chemiluminescence response are induced by the C5b-9 insertion to their cell membranes but not by the deposition of C3.[31]

From a functional point of view, it was also demonstrated by the present study that complement played a pivotal role in the functional impairment of the proximal tubules associated with proteinuria. In group I rats, inulin clearance was significantly reduced by approximately 80% and PAH clearance by 50%. When serum complement was depleted by CVF (group II), only PAH clearance returned to the normal level at day 7 whereas inulin clearance of this group still remained at a lower level. Significant reduction of inulin clearance by PAN was reported previously, and the mechanism for this was postulated to be direct toxicity of PAN for the glomerular visceral epithelial cells. This is thought to be a complement-independent process. In contrast, it was demonstrated in the present work that the reduction of PAH clearance, which indicates impairment of proximal tubular cell function, was complement dependent. Thus, it is highly probable that abnormal glomerular barrier function due to nonimmunological mechanisms can induce com-

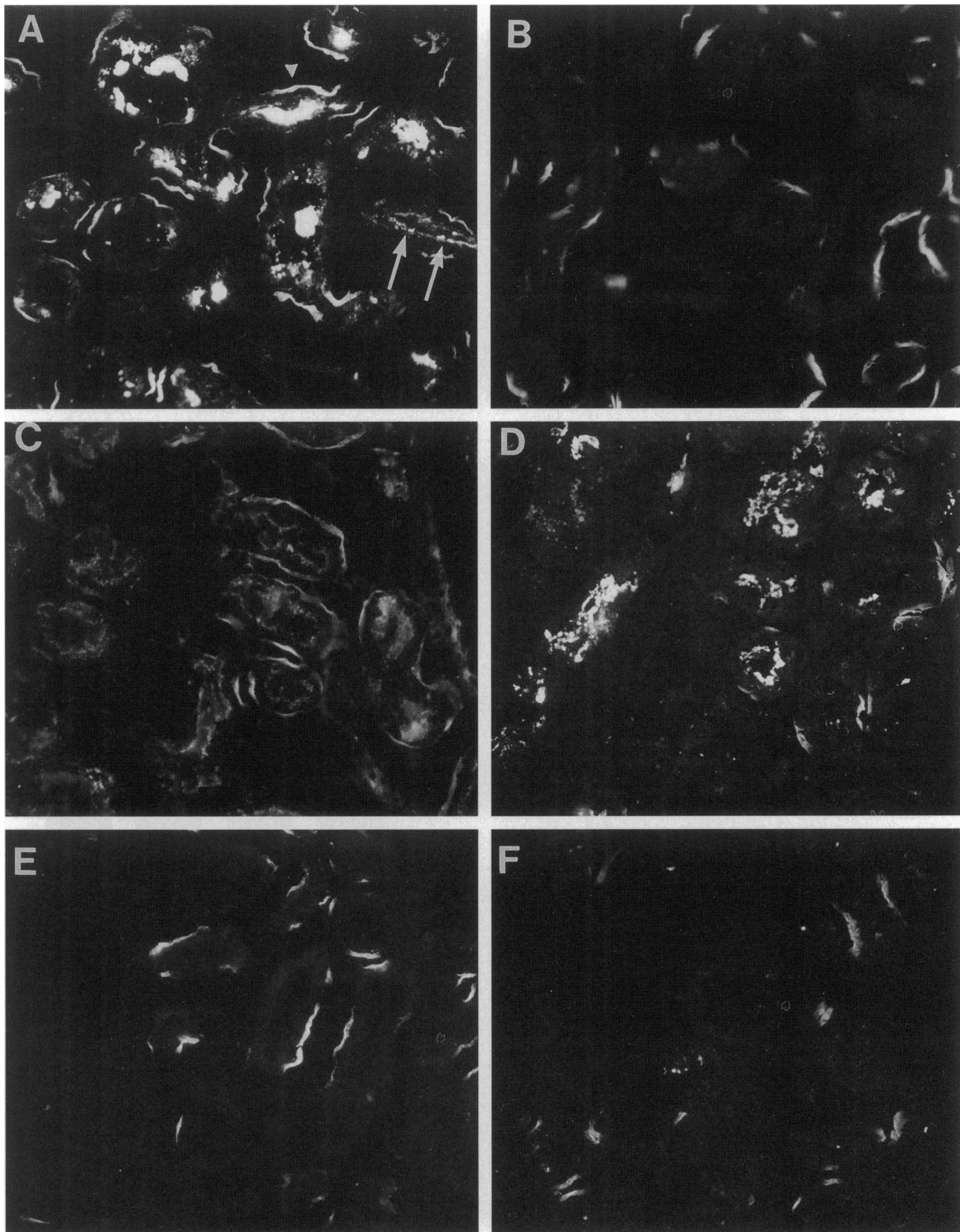

Figure 6. Immunofluorescence microscopy of C3 (**A** to **C**) and C5b-9 (**D** to **F**) in renal cortex. Magnification, ×400. **A** and **D**: Group I. **B** and **E**: Group II. **C** and **F**: Group III. In group I rats, deposition of C3 and C5b-9 in the luminal surface of proximal tubules was observed. In group II, there was no deposition of complement component. In group III rats, deposition of C3 was observed although C5b-9 was absent. The degree of C3 deposition was milder in group III than in group I. **Arrows** and **arrowheads** indicate tubular brush border and tubular basement membrane, respectively.

plement-dependent tubulointerstitial injury and further functional impairment of the kidney.

From the therapeutic point of view, anti-complement reagents that could be used for clinical treatment have been studied by many investigators. Those efforts have not been very successful until recently. Human complement receptor type 1 (CR1)[32,33] is a molecule that is expressed mainly on blood-borne cells and podocytes of the glomerulus and have both complement regulatory activity at the level of C3 and complement receptor activity.[27] Recently a soluble form of recombinant human CR1 (sCR1) was genetically synthesized, and its comple-

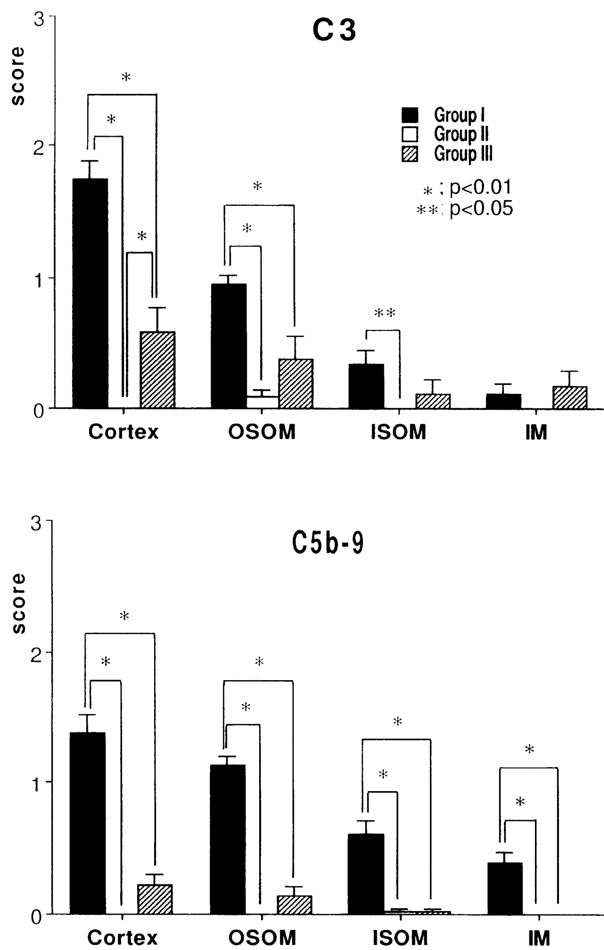

Figure 7. Quantitative measurement of complement deposition along the luminal surface of tubular cells. In rats of Group I, C3 and C5b-9 were detected mainly in cortex and outer stripe. In group II, deposition of C3 and C5b-9 was not detected at all. In group III, C3 was detected, but the extent of staining was significantly less prominent than in group I. Deposition of C5b-9 was not significant in group III.

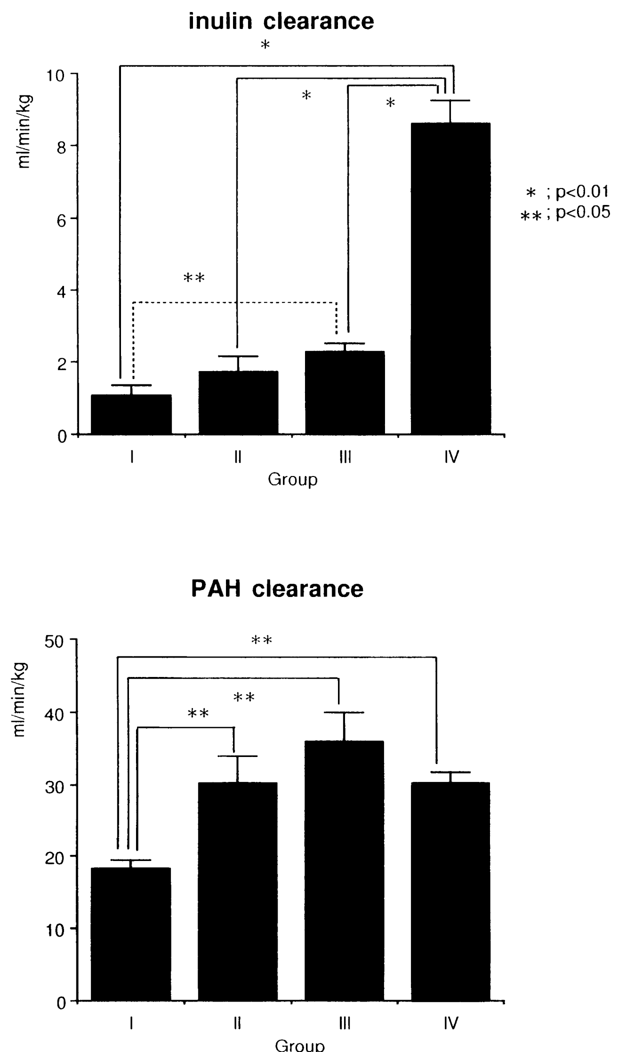

Figure 9. The effects of serum complement depletion and sCR1 on the renal function in PAN rats. Inulin clearance and PAH clearance was significantly reduced in group I rats. Only PAH clearance was improved to the normal level in groups II and III rats, although inulin clearance still remained at a significantly low level.

ment inhibitory activity was successfully reported in some experimental models including Arthus reaction,[17] post-ischemic heart injury,[16] reperfusion injury of skeletal muscle,[34] and antibody-mediated experimental glomerulonephritis.[35] As sCR1 inhibits complement activation by decay acceleration of C3 convertase and cleavage of the C3b molecule with factor I, there is no further activation of complement. In addition, sCR1 is a protein of human origin, and it does not induce antibody production in man. In the present study, sCR1 was very effective in reducing the proteinuria-induced tubulointerstitial injury and functional impairment of the diseased kidney. In group III rats of this study, immunohistochemical examination revealed that only trace amounts of C5b-9 were detected whereas C3 was present on the apical side of proximal tubules. Furthermore, sCR1 was detected in the urine of group III rats. No complement deposition was seen in glomerulus. These data show that injected sCR1 was filtered into the tubular lumen and inhibited complement activation *in situ*.

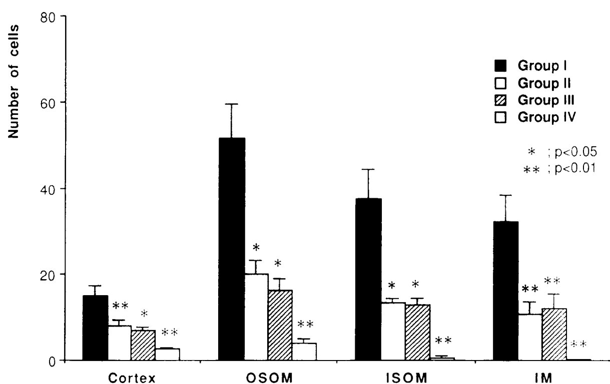

Figure 8. Macrophage infiltration into the renal interstitial tissue. In rats of group I, significant numbers of ED1-positive cells were present mainly in the outer medulla. Although macrophage infiltration is still significantly high in rats of groups II and III compared with group IV, the degree of macrophage infiltration was significantly less prominent in groups II and III rats than in group I.

In summary, the present work demonstrated *in vivo* for the first time that complement mediates proteinuria-associated tubulointerstitial injury. Depletion or inhibition of complement significantly improved tubular injury induced by proteinuria. As the improvement of histological damage by these procedures was not complete, the mechanisms other than complement activation might have partially contributed to the development of proteinuria-associated tubulointerstitial injury. As reported, tubulointerstitial damage is better correlated with the progression of renal failure, and tubulointerstitial injury is often seen in the proteinuric patients. The control of complement activation might be an important strategy for the prevention and treatment of progressive renal diseases associated with persistent proteinuria.

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