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Crry, a complement regulatory protein, modulates renal interstitial disease induced by proteinuria[1]

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Crry, a complement regulatory protein, modulates renal interstitial disease induced by proteinuria.

Background. Recent studies have suggested a role for urinary complement components in mediating tubulointerstitial damage, which is known to have a good correlation with progression of chronic renal diseases. Although accumulating evidence suggests that complement regulatory proteins play an important protective role in glomeruli, their role in renal tubules remains unclear. In order to establish the role of a complement regulatory protein, Crry, in renal tubular injury, we employed a molecular biological approach to block the expression of Crry in tubules of animals with proteinuria induced with puromycin aminonucleoside nephritis (PAN).

Methods and Results. Two different antisense oligodeoxynucleotides (ODNs) against Crry were designed and applied to cultured rat mesangial cells *in vitro* in order to establish their efficacy. Antisense ODN treatment resulted in decreased expression of Crry protein associated with increased sensitivity to complement attack in cell lysis assays compared with control ODN treatment or no treatment (44.7, 1.50, and 1.34%, respectively). Antisense ODNs did not affect the expression of Thy1 as a control, confirming the specificity of our ODNs. *In vivo*, we performed selective right renal artery perfusion to administer antisense ODNs to the kidney and showed prominent uptake of ODNs by proximal tubular cells. Reduced expression of Crry protein was demonstrated in proximal tubular cells in antisense ODNs-treated kidneys. Normal rats treated with the antisense ODNs did not show any pathological changes. However, in PAN, rats with massive proteinuria showed increased deposition of C3 and C5b-9 in tubules in antisense-treated kidneys, and histological assessment revealed more severe tubulointerstitial injury in antisense-treated animals compared with controls.

Conclusion. These results establish a pathogenic role for complement in leading to tubulointerstitial injury during proteinuria and, to our knowledge for the first time, show a protective role of a complement regulatory protein, Crry, in renal interstitial disease.

[1] **See Editorial by Quigg, p. 2315**

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In a variety of kidney diseases, renal prognosis is better correlated with the structural damage in the tubulointerstitium than in the glomerulus [1–13]. Accumulating evidence suggests that proteinuria is also well correlated with tubulointerstitial injury and that proteinuria may itself be an independent mediator of progression of renal diseases [14–20, reviewed in 21–24].

Proteinuria can induce tubulointerstitial injury by several mechanisms. High protein concentrations in tubular fluid, *per se*, can be toxic to tubular cells by leading to lysosomal swelling or obstructing tubules with proteinaceous casts [25]. Specific proteins that have been reported to be cytotoxic include transferrin [26, 27] and lipoproteins [28, 29], which can generate oxidants or chemotactic factors. However, recent studies of complement-depleted rats emphasized roles of complement components contained in excessive proteinuria in tubulointerstitial injury [30, 31]. Tubular damage induced chronically by complement activation may result in deterioration of renal function over time.

The organism has developed a complex system of proteins that regulate inappropriate complement activation and prevent tissue injury, so-called complement regulatory proteins [32–37]. Crry is a rodent complement regulatory protein that serves the mechanisms of both decay-accelerating factor (DAF) and membrane cofactor protein (MCP) [38, 39]. cDNA of rat Crry was recently cloned [40, 41]. Proximal tubular cells in rats are endowed with some complement regulatory proteins, including Crry [34, 42], although their functional roles remain unclear.

Therefore, we performed this study to elucidate the role of a complement regulatory protein, Crry, in tubules utilizing an antisense oligodeoxynucleotide (ODN) ap-

proach. Our findings provide the first evidence, to our knowledge, that complement regulatory proteins play an important role in modulating interstitial injury secondary to proteinuria.

METHODS

Mesangial cell culture and transfection

Mesangial cells were obtained by culturing glomeruli isolated from kidneys of 200 to 250 g male Wistar rats by a conventional sieving method [43, 44]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Wako Pure Chemical Industries, Osaka, Japan).

Cells were transfected with ODNs as described later in this article using cationic liposomes (LipofectAMINE Reagent; Life Technologies, Inc., Gaithersburg, MD, USA). In brief, 1.0×10^5 cells per well were seeded in a 12-well tissue culture plate and were incubated until the cells were approximately 80% confluent. To allow ODN–liposome complexes to form, 1.0 μg of ODNs and 8 μl of LipofectAMINE reagent were combined in 400 μl serum-free medium and incubated at room temperature for 30 minutes. Then 400 μl/well of the complex solution were overlayed onto the cells rinsed with serum-free medium. After three hours of incubation, the medium was replaced with that containing 20% FCS. Forty-eight hours later, the cells were applied to subsequent experiments.

Synthesis of oligodeoxynucleotides

Synthetic ODNs employed in this study were designed from the sequences of the rat Crry [40]. Antisense ODN AS1, consisting of the sequence 5′-CAGAGGCGAA GAAGCCTCCAT-3′, was the inverse complement of the positions 24 to 44. Another antisense ODN AS2, consisting of the sequence 5′-TACAAGGCGCCCCA CGGGGTC-3′, was the inverse complement of the positions 45 to 65. Sense ODN S1 consists of the sequence 5′-ATGGAGGCTTCTTCGCCTCTG-3′. The sequence of scrambled ODNs based on AS1, SCRA, was 5′-GCAA CACGTATAGGCACGGAC-3′. Biotinylated S1, Bio-S1, was biotinylated on the 5′ end of S1. These sequences did not show significant homology with any other sequences in the nucleic acid sequence database in GenBank. These ODNs were synthesized on a PerSeptive Biosystems synthesizer (PerSeptive Biosystems, Inc., Framingham, MA, USA) and were purified over a NAP-10 column (Pharmacia Biotech AB, Uppsala, Sweden). All of them were phosphorothioated.

Antibodies

The characteristics of 5I2 monoclonal antibody to Crry have been described elsewhere [45]. The characteristics of 2A1 monoclonal antibody to rat C5b-9 have also been described previously [46]. Polyclonal goat antithymocyte serum (ATS; anti-Thy1) IgG, which was used for complement-mediated cell lysis assay, was purified using a caprylic acid precipitation method [47, 48]. For Western blotting, a hybridoma line producing monoclonal anti-Thy1 antibody (OX-7) was purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK), and then OX-7 was purified as Yagi et al described [49]. Monoclonal antibody against vimentin (clone V9) was purchased from Dako Corp. (Carpinteria, CA, USA).

Western blot analysis

To detect Crry protein, Western blotting was performed using the 5I2 antibody against Crry. Mesangial cells were washed with phosphate-buffered saline (PBS) twice and then solubilized for 30 minutes at room temperature in cell-solubilizing and reducing buffer. The protein concentrations of the samples were first measured by DC protein assay (Bio-Rad, Hercules, CA, USA). Ten micrograms of protein were carefully loaded in each lane, and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under nonreducing conditions. The bound antibody was detected with 1 μg/ml of alkaline phosphatase-conjugated antimouse IgG (Promega, Madison, WI, USA). 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma Fast; Sigma Chemical Co., St. Louis, MO, USA) were used as a substrate. The gel was stained with Commassie brilliant blue, and all of the lanes of the stained gel were confirmed to be equivalent by visual inspection.

Complement-mediated cell lysis assay

To determine the functional significance of transfecting cells with the antisense ODNs against Crry cDNA, complement-mediated cell lysis assay was performed. Mesangial cells (approximately $10^4/cm^2$) were passaged into 12-well plates and were grown until 50 to 80% confluent. The cells were transfected as described earlier in this article, and after 48 hours of incubation, the transfected cells were washed twice with PBS. The nontransfected cells that were grown until almost confluent were used as a control. The cells were sensitized with 300 μl of anti-Thy1 (ATS) antibody (2.0 mg/ml) for 20 to 30 minutes at room temperature. After further washing, the cells were incubated with 400 μl of 5 to 20% rat serum as a complement source for two hours at 37°C together with DMEM. Cell lysis was quantitated by lactate dehydrogenase (LDH) assay kit (Wako Pure Chemical Industries) as follows: Kinetic determination of LDH was based on the spectrophotometric method of Wroblewski and LaDue [50]. The activity of LDH was measured by the reductive ability of NADH, which was produced as

lactate and was oxidized to pyruvate. The background value was estimated using cells incubated with serum without sensitization. After the assay, all cells were lyzed with 2% Triton X-100 (Sigma Chemical Co.), and the percentage of complement-mediated cell lysis was calculated.

Experimental animals

Male Wistar rats weighing 180 to 200 g were purchased from Nippon Seibutsu Zairyo Center Co., Ltd. (Saitama, Japan). All animal studies were performed in an accredited animal care facility according to the Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

Induction of the puromycin aminonucleoside nephritis model

The puromycin aminonucleoside nephritis (PAN) model was induced in rats by single injection of 5 mg/100 g body wt puromycin (Wako) via tail vein.

Experimental protocol

On day 0, puromycin (5 mg/100 g body wt) was injected into rats. On day 4 when proteinuria had developed, we performed selective right renal artery perfusion through the superior mesenteric artery with 0.2 μg of antisense or sense ODNs in 2 ml of PBS as described previously [51]. Rats were housed in metabolic cages with free access to water, and urine was collected overnight on days 3 and 4, 6 and 7, and 11 and 12 for measurement of urinary protein. Blood samples were obtained from the tail vein on day 7 and via cardiac puncture on day 12 for the measurement of blood urea nitrogen (BUN). On day 7, a survival right renal biopsy was performed, and on day 12, a sacrificial right renal biopsy was performed for histological analysis.

Renal histology and immunohistochemistry

Tissue for frozen sections was embedded in OCT (Lab-Tek Products; Miles Laboratories, Naperville, IL, USA) and were snap frozen in acetone in liquid nitrogen. Frozen sections were stained for Crry and biotinylated ODNs using the Vectastain ABC-AP Kit (Vector Laboratories, Inc., Burlingame, CA, USA) with Vector Red (Vector Laboratories) as substrate. For vimentin, 4 μm sections of Methyl Carnoy's fixed tissue were stained with clone V9 using a DAB detection system (HistoFine SAB-PO Kit; Nichirei, Tokyo, Japan).

Immunofluorescence study

To examine the tubulointerstitial deposition of complement, 4 μm frozen sections were stained with FITC-conjugated goat antibodies against rat C3 (Cappel, Organon Teknika Corp., Durham, NC, USA) for C3. For C5b-9, we used a mouse monoclonal antibody 2A1 against rat C5b-9, which was then detected by biotinylated anti-mouse IgG (secondary antibody; Dako Corp.) and Oregon green/Neutralite Avidin (Molecular Probes Inc., Eugene, OR, USA). The fluorescent intensity of C3 staining was quantitated by computed analysis with the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA, USA). Briefly, the average of the pixel gray scale values (0 to 4095) contained in the objective was calculated (Σ gray scale value/number of pixels). Five randomly selected separate fields of tubulointerstitium were assessed in each section, and the fluorescence intensity was expressed as the mean compensated by background intensity from nontreated normal rat kidney sections. C5b-9 deposition was quantitated as the total number of positive tubules in the four randomly selected separate fields of tubulointerstitium in each section.

Histological assessment of tubulointerstitial injury

To examine the renal histology, 4 μm sections of paraffin-embedded tissues were stained with periodic acid-Schiff reagent (PAS). In each biopsy, the degree of tubulointerstitial injury was evaluated in a blinded fashion on the PAS-stained sections. As a semiquantitative analysis, the total number of the tubules with dilation and degeneration, including vacuolar changes, loss of brush border, detachment of tubular epithelial cells, and condensation of tubular nuclei, was counted in 10 randomly selected cortical fields (×400) of each section.

Measurement of proteinuria and blood urea nitrogen

Urinary protein excretion of the rats was measured by DC protein assay (Bio-Rad). Measurement of BUN was performed by urease-indophenol method with Urea N B (Wako Pure Chemical Industries).

Statistical analysis

All values are presented as mean ± SD. Statistical comparisons were analyzed with the program StatView (Abacus Concepts, Berkeley, CA, USA) using the analysis of variance followed by the Bonferroni/Dunn method for multiple group comparisons and paired or unpaired Student's *t*-test as appropriate. A P value of less than 0.05 was considered statistically significant.

RESULTS

Expression of Crry protein in transfected mesangial cells with antisense

To examine the efficacy of our antisense ODNs, we employed cultured rat mesangial cells, in which the complement-mediated cell lysis assay had been established [52, 53]. To determine the expression levels of Crry protein in mesangial cells transfected with ODNs, Western blot analysis was performed. The total amount of Crry was significantly decreased in cells transfected with two different antisense ODNs compared with those trans-

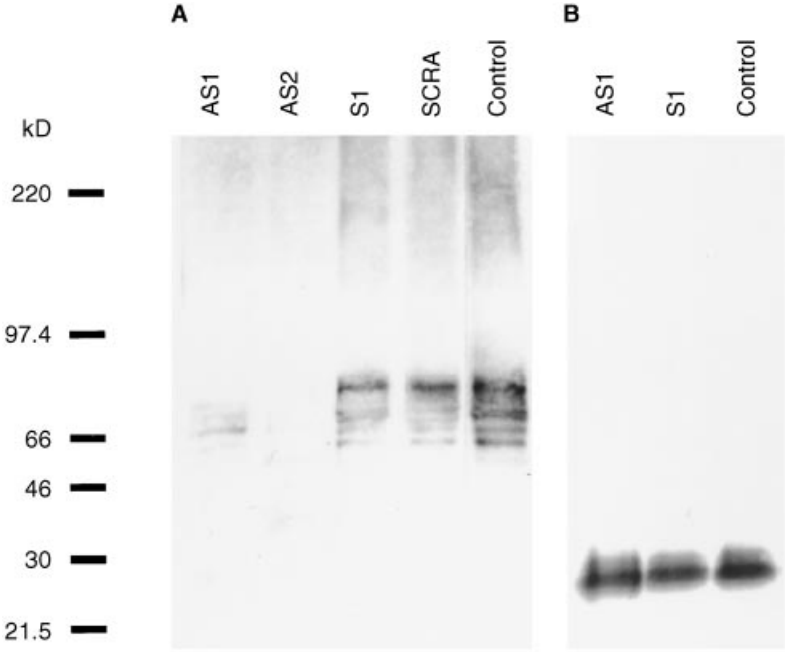

Fig. 1. Western blot analysis comparing the expression of Crry protein in normal and ODNs-transfected rat mesangial cells. Rat mesangial cells transfected with antisense ODNs (AS1 and AS2), sense ODNs (S1), scrambled ODNs (SCRA), or nontransfected control cells (control) were examined in order to investigate the efficacy of the ODNs. The total amount of Crry is markedly decreased in cells treated with AS1 or AS2, compared with those with S1, SCRA, or control (*A*). In contrast, Western blotting with OX-7 revealed an equal amount of thymocyte antigen between AS1, S1, and control, ruling out a nonspecific effect of antisense ODNs to reduce the expression of cellular proteins (*B*). Similar results were obtained in four separate experiments. Markers are expressed in kilodaltons (kD).

fected with sense or scrambled ODNs (Fig. 1A). In contrast, Western blotting with OX-7 showed an equal amount of the thymocyte antigen in antisense ODNs and sense ODNs-treated cells, ruling out a toxic effect of antisense ODNs to reduce the expression of cellular proteins nonspecifically (Fig. 1B).

Augmented complement-mediated cell lysis in antisense treated cells

Prior to performing a cell lysis assay, the nonspecific cell toxicity of the ODNs was examined. The supernatants of the transfected cells were assessed by measuring LDH. There was no significant difference among antisense, sense, and scrambled ODNs (data not shown).

To examine the functional characteristics of antisense-transfected cells, we performed complement-mediated cell lysis assays. Mesangial cells sensitized with ATS IgG demonstrated cell lysis that was dependent on the concentrations of rat serum used as a complement source as described previously [52, 53]. When sensitized normal mesangial cells were incubated with 5% normal rat serum, no more than 2.5% complement-mediated cell lysis was observed (1.34 ± 1.09%; 4 separate experiments were performed in triplicate). A decrease of Crry expression by antisense ODNs was expected to increase sensitivity of the cells to complement attack, and we employed sublytic conditions using 5% rat serum here. Sensitized mesangial cells transfected with AS1 were much more susceptible to complement attack than controls transfected with S1 (AS1, 44.7 ± 6.89%; S1, 1.50 ± 1.47%, $P < 0.05$; Fig. 2).

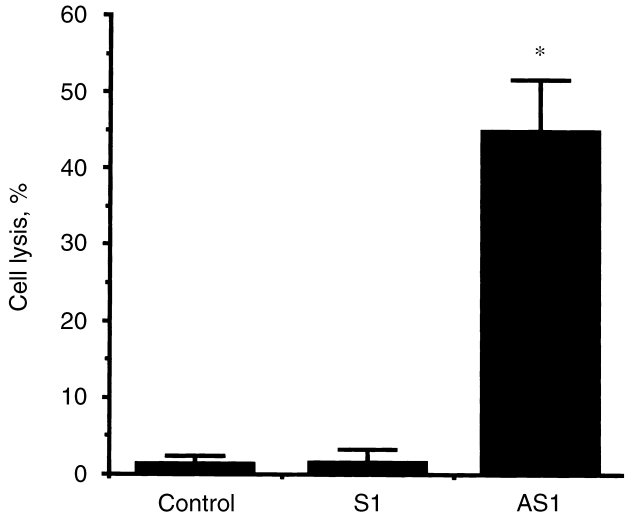

Fig. 2. Complement-mediated cell lysis assays of oligodeoxynucleotides (ODNs)-transfected mesangial cells. Transfected cells were subjected to complement-mediated lysis by treatment of cells sensitized with ATS with 5% normal rat serum as a complement source. This caused no more than 2.5% cell lysis in untreated mesangial cells (control). Cell lysis was determined as LDH release after 1.5 hours of incubation at 37°C. Note the marked sensitivity of the antisense-treated cells (AS1) to complement attack compared with sense-transfected cells (S1). *$P < 0.05$.

Uptake of oligodeoxynucleotides in tubular cells by selective renal artery perfusion

In previous studies, the administration of ODNs via the circulation resulted in the localized deposition of ODNs in proximal tubules [54–57]. To ensure this localization, we performed selective right renal artery perfusion with

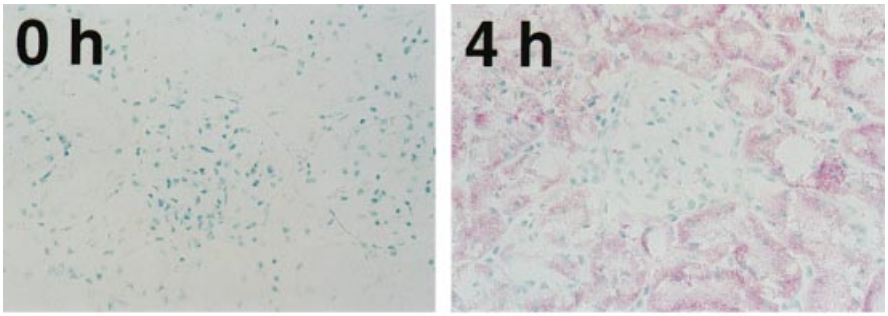

Fig. 3. Uptake of ODNs in tubular cells at 4 and 24 hours after selective renal artery perfusion. Biotinylated sense ODNs, Bio-S1 (0.2 μg), were perfused in normal rat kidney and biopsies were obtained 4 and 24 hours later. Uptake of Bio-S1 at both intervals was confined primarily to proximal tubules. Staining was not observed in other segments of nephron. (×200)

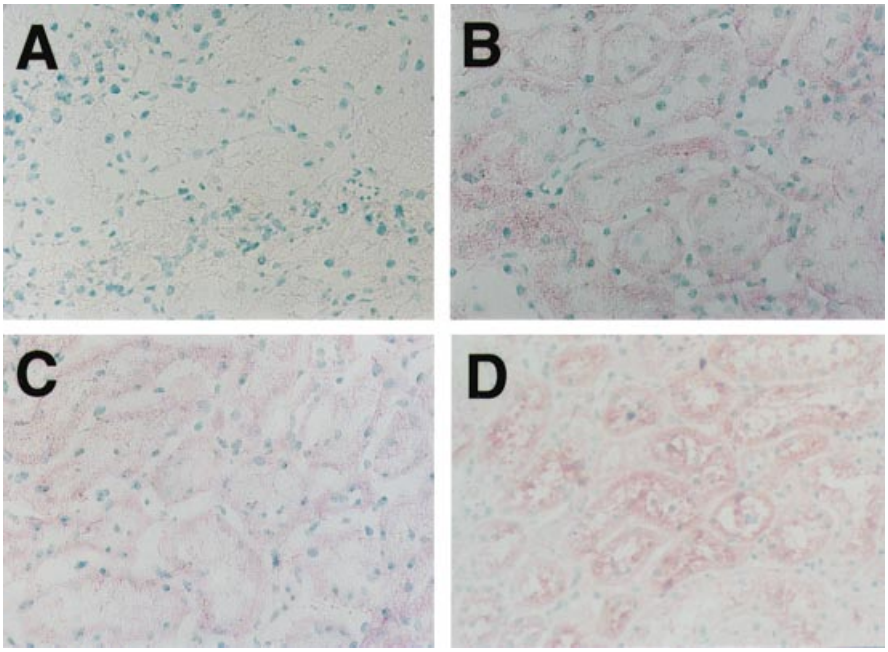

Fig. 4. Reduced expression of Crry protein in proximal tubular cells transfected with antisense ODNs. In three days after the selective right renal artery perfusion, Crry was not detectable in proximal tubules in the kidneys perfused with 0.2 μg of AS1 (*A*), whereas sense-perfused samples (*B*) did not show any changes of Crry expression levels, compared with sections of untreated control kidneys (*C*). The expression levels of Crry in antisense treated kidneys returned to normal seven days after the treatment (*D*). (×200)

Table 1. Urinary protein excretion and blood urea nitrogen (BUN) in antisense and sense groups of PAN rats

	Antisense (AS1)	Sense (S1)	*t*-test
Day 7			
Urinary protein *mg/day*	44.9 ± 14.7	31.7 ± 14.5	NS
BUN *mg/dl*	18.7 ± 2.06	17.4 ± 1.45	NS
N	14	13	—
Day 12			
Urinary protein *mg/day*	124 ± 33.8	105 ± 33.6	NS
BUN *mg/dl*	25.7 ± 2.34	20.5 ± 2.03	NS
N	11	9	—

Data are mean ± SD. NS is not significant.

biotinylated ODNs, Bio-S1. Bio-S1 (0.2 μg) was perfused in normal rat kidneys, and biopsies were obtained 4 and 24 hours later. Proximal tubular cells were positive for ODNs deposition in four hours, whereas glomerular cells and other segments of the nephron were negative (Fig. 3). At 24 hours, the level of staining remained high in proximal tubular cells.

Reduced expression of Crry protein in proximal tubular cells by antisense oligodeoxynucleotides

To determine the effect of antisense ODNs on the expression of Crry protein *in vivo*, selective right renal

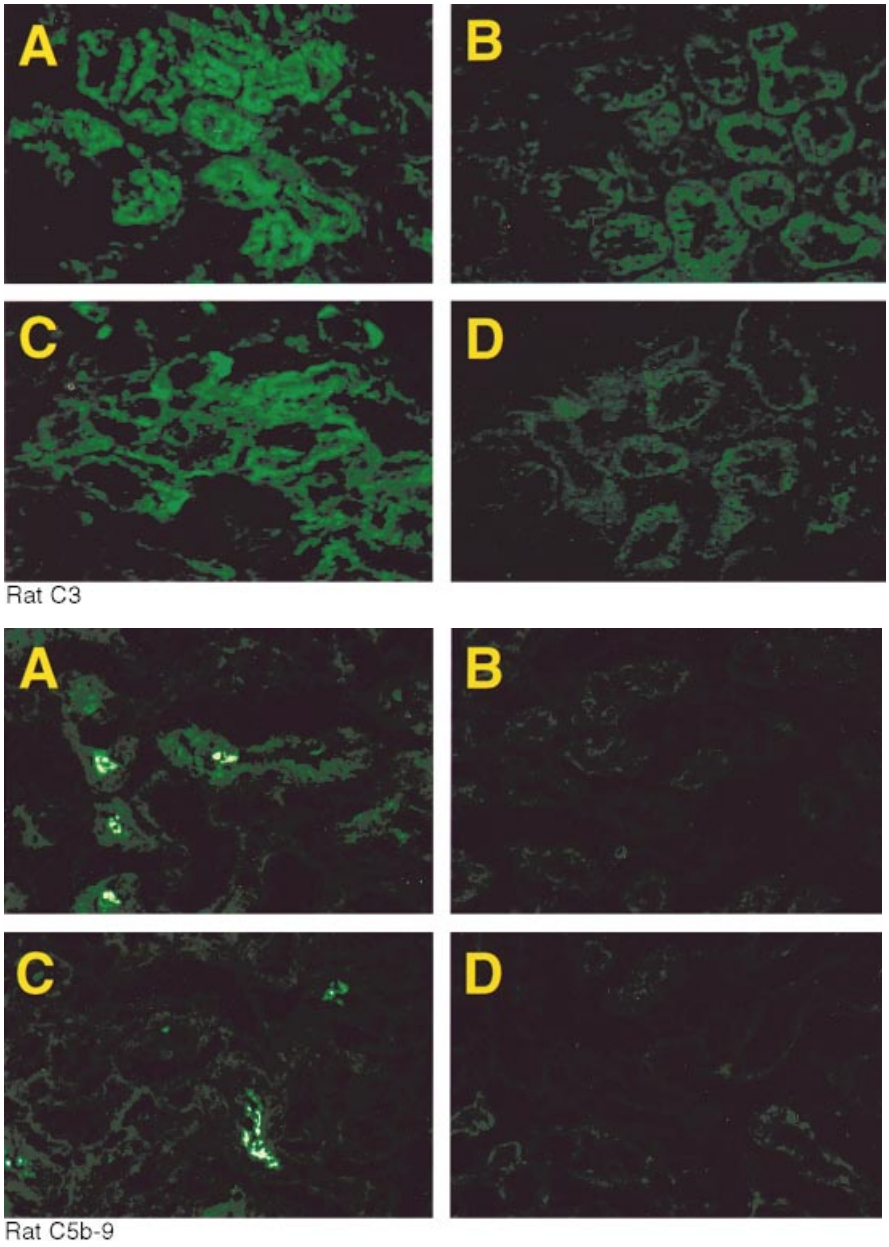

Fig. 5. Immunofluorescence findings. Deposition of rat C3 (top panels) and C5b-9 (bottom panels) in antisense (*A* and *C*) and sense (*B* and *D*) treatment in PAN rats is shown. Deposition of rat C3 and C5b-9 mainly along the luminal side of proximal tubules was clearly demonstrated in antisense-treated kidneys at day 7 (*A*) and at day 12 (*C*) in contrast to control sense-treated kidneys at day 7 (*B*) and at day 12 (*D*), respectively. (×400)

artery perfusion of ODNs was performed in normal rats. Antisense ODNs (AS1) and sense ODNs (S1; 0.02, 0.06, and 0.2 μg) were perfused. Careful examinations of the biopsy samples stained by PAS and hematoxylin and eosin (HE) revealed no significant pathological changes in either group, and we decided to use 0.2 μg of ODNs in the following studies, expecting the maximal effects without nonspecific damage on tissues. Immunohistological analysis with monoclonal antibody against Crry, 5I2, revealed that Crry was not detectable in proximal tubules in the kidneys perfused with 0.2 μg of AS1 at one day and three days, whereas sense perfused samples at any dose did not show any changes in Crry expression levels compared with sections of untreated control kidneys (Fig. 4). The expression levels of Crry in antisense treated kidneys returned to normal seven days after the perfusion (Fig. 4).

Augmented tubulointerstitial injury by antisense oligodeoxynucleotides against Crry in PAN rats

Induction of the PAN model. To evaluate whether or not tubulointerstitial injury associated with massive proteinuria was affected by proximal tubule localization of antisense ODNs against Crry, we used the PAN model of nonimmunologically-induced nephrotic syndrome. On day 4, no rats developed proteinuria. On day 7, urinary

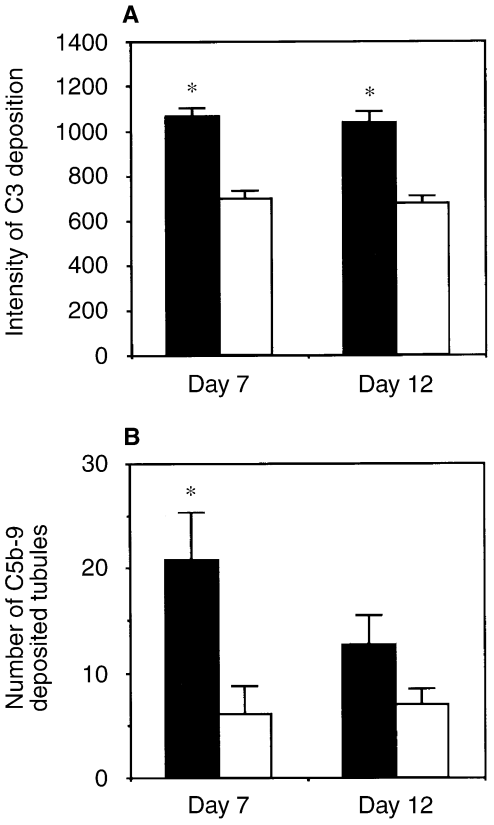

Fig. 6. Quantitative analysis of C3 and C5b-9 deposition in antisense- or control sense-treated kidneys of PAN rats. The degree of C3 deposition was quantitated by computed analysis. C5b-9 deposition was quantitated as the total number of positive tubules in four separate fields of tubulointerstitium in each sample. The intensity of fluorescence for C3 (*A*) was significantly increased in the antisense (AS1)-treated (■) kidney sections on day 7 and day 12 as compared with those of sense (S1; □). Also, the number of C5b-9 (*B*)-positive tubules was remarkably increased in antisense groups (■) on day 7 and day 12 as compared with sense groups (□). $^*P < 0.05$.

protein levels in the antisense group and sense group were 44.9 ± 14.7 and 31.7 ± 14.5 mg/day, respectively ($P > 0.1$), and on day 12, 124 ± 33.8 and 105 ± 33.6 mg/day, respectively ($P > 0.1$; Table 1). Therefore, the amount of complement components in urine was assumed to be similar in both groups. Also, BUN was in the normal range at day 7 and at the upper limit of normal at day 12 in both groups, with no significant difference ($P > 0.1$; Table 1). For immunohistochemical and histological analysis, the number of rats in each group was as follows: antisense group at day 7 and day 12, $N = 14$ and $N = 11$, respectively; sense group at day 7 and day 12, $N = 13$ and $N = 9$, respectively.

Immunohistological findings. Immunofluorescence study revealed the strong deposition of C3 in cortical tubules on day 7 and on day 12 in the antisense groups as compared with control sense groups (Fig. 5a). By computed analysis, the degree of C3 deposition was quantitated. The intensity of fluorescence for C3 was significantly increased in the antisense-treated kidney sections both on day 7 and on day 12 as compared with those of sense (AS1 and S1 treated at day 7, 1070 ± 39.1 and 698 ± 37.5, respectively, $P < 0.05$; AS1 and S1 treated at day 12, 1040 ± 51.0 and 676 ± 33.7, respectively, $P < 0.05$; Fig. 6A). C5b-9 deposition was quantitated as the total number of positive tubules in four separate fields of tubulointerstitium in each sample. The number of C5b-9–positive tubules was also increased in the antisense groups on day 7 and day 12 as compared with sense groups (Fig. 5b), although the difference between the two groups reached statistical significance only at day 7 (AS1 and S1 treated at day 7, 20.8 ± 4.63 and 6.08 ± 2.72, respectively, $P < 0.05$; AS1 and S1 treated at day 12, 12.7 ± 2.73 and 7.00 ± 1.54, respectively, $P = 0.10$; Fig. 6B).

PAS staining. Histological assessment of tubulointerstitial injury was performed with PAS staining. Tubules with dilation and/or degeneration, including tubular hyaline casts, loss of brush border, and detachment of tubular epithelial cells from basement membrane, were more often observed in antisense-treated kidneys compared with control sense-treated kidneys (Fig. 7a). Semiquantitative analysis revealed that the extent of tubular dilation and/or degeneration was more severe at both day 7 and day 12 in antisense groups (AS1 and S1 treated at day 7, 15.7 ± 2.02 and 7.15 ± 0.876, respectively, $P < 0.05$; AS1 and S1 treated at day 12, 15.5 ± 1.64 and 7.33 ± 1.31, respectively, $P < 0.05$, Fig. 7b).

Vimentin staining. Vimentin staining was performed as another indicator of tubulointerstitial injury. More vimentin-positive tubules were observed in tubules in the cortex at day 7 and day 12 in the antisense-treated rats than in the control animals, although the difference between the two groups did not reach statistical significance (AS1 and S1 treated at day 7, 18.5 ± 5.93 and 6.77 ± 3.22, respectively, $P = 0.10$; AS1 and S1 treated at day 12, 20.4 ± 4.43 and 10.6 ± 3.43, respectively, $P = 0.10$). Staining for vimentin in glomeruli and renal vasculature was identical in the two groups.

DISCUSSION

Although structure–function correlations between disturbances in glomerular structure and kidney failure are relatively imperfect [2, 3], distinct relationships between tubulointerstitial damage and impairment of kidney function have been well established [1, 4–13]. The cause of progressive loss of renal function by tubulointerstitial injury is likely to be multifactorial. Tubular atrophy may lead to a reduction in the glomerular filtration rate (GFR) through tubuloglomerular feedback by increasing fluid delivery to the macula densa [7]. Interstitial fibrosis may occlude postglomerular capillaries and reduce glomerular

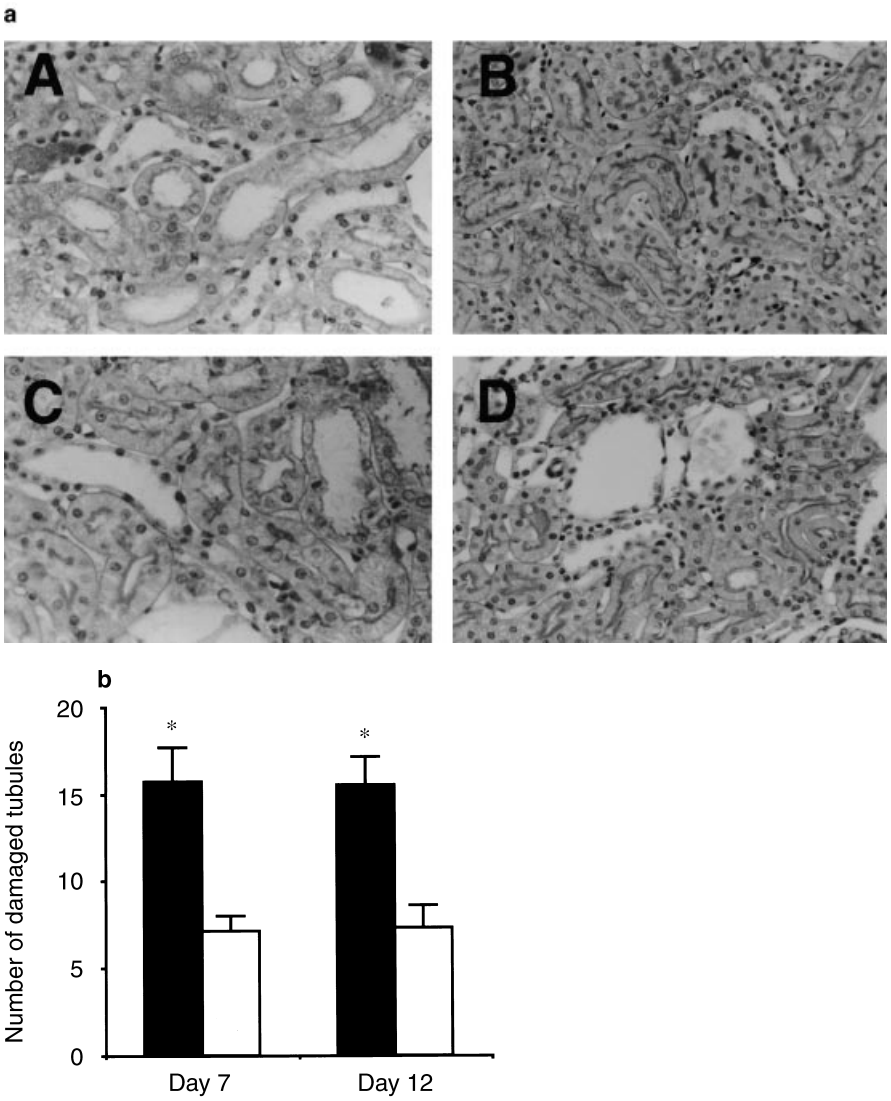

Fig. 7. Histological assessment of tubulointerstitial injury. (*a*) PAS staining of antisense- (*A* and *C*) or sense- (*B* and *D*) treated kidney at day 7 (*A* and *B*) and at day 12 (*C* and *D*) in PAN rats. Tubules with dilatation and/or degeneration were more often observed in antisense-treated kidneys compared with control sense-treated kidneys (×200). (*b*) Semiquantitative analysis of tubulointerstitial injury. The extent of tubular dilatation and/or degeneration was significantly more severe in the antisense groups both at day 7 and day 12 as compared with control sense groups. Symbols are: (■), antisense (AS1)-treated; (□), sense (S1)-treated. $*P < 0.05$.

blood flow [6]. Tubular damage may cause atubular glomeruli and a decrement in the number of functional nephrons [58, 59].

Considerable evidence suggests that proteinuria itself may give rise to tubulointerstitial injury, serving as an independent mediator of progression of chronic renal failure [reviewed in 21–24]. One of the potential mechanisms by which proteinuria causes tubulointerstitial nephritis is activation of complement components contained in proteinuric urine [30, 31]. Although complement components are not filtered by glomeruli under normal conditions, their presence has been demonstrated in the urine of patients with nephrotic syndrome [60, 61]. These complement components are contained in massive proteinuria deposits on renal tubules [62] and may be activated by the brush border, which was shown to activate the alternative pathway directly [63, 64]. Complement activation on the tubular epithelial surface can result in cytoskeletal alterations and the generation of inflammatory mediators, which may contribute to tubulointerstitial damage [65]. Micropuncture studies demonstrating that inactivation of complement prevented proximal tubular lesions induced by intraluminal perfusion with serum [66] supported this hypothesis. Recent articles by Morita et al and Nomura et al clearly established the pathogenic role of complement components in proteinuric urine. Complement depletion with cobra venom factor or inhibition with recombinant soluble human complement receptor type 1 dramatically ameliorated tubulointerstitial injury induced by proteinuria in rats with aminonucleoside nephrosis or with mesangial proliferative glomerulonephritis [30, 31].

Although important roles of complement regulatory proteins in glomerular cells have been well established [52, 53, 67–73, reviewed in 74], their roles in tubular cells remain to be determined. We hypothesized that

complement regulatory proteins in renal tubular cells might protect tubules from attack by complement components derived from proteinuric urine. To investigate the role of one complement regulatory protein, Crry, in tubules, we employed antisense ODNs to reduce Crry expression [75–79]. Recent kinetic studies showed that when phosphorothioated ODNs were administered systemically, a major portion of the dose was taken up by the proximal tubules in the kidney [80–82]. Furthermore, electrophoresis of the isotope-labeled ODNs administered intravenously showed that ODNs retained intact in the kidney for at least four days after infusion [54, 55]. These studies strongly suggest that the kidney, especially renal tubules, is an ideal target for *in vivo* antisense ODN experiments. We ensured the specific localization of ODNs by selective right renal artery perfusion with biotinylated ODNs (Fig. 3).

To examine the efficacy and specificity of the antisense ODNs, we performed *in vitro* experiments utilizing cultured rat mesangial cells first. Mesangial cells were suitable for this purpose because they constantly express Crry protein on the cell membrane [42, 70], and complement-mediated cell lysis assays to investigate the functional consequences of the antisense ODNs have been well established in these cells [52, 53]. Western blot analysis and complement-mediated cell lysis assays clearly demonstrated the specificity and functional efficacy of our antisense ODNs (Figs. 1 and 2).

Previous immunohistochemical studies as well as our study demonstrated expression of Crry in tubular cells in rats [42]. Our *in vivo* antisense treatment by selective renal artery perfusion successfully decreased Crry expression in proximal tubules (Fig. 4). However, a reduction in Crry expression levels in normal rats did not induce any pathological changes in our studies. In contrast, Nomura et al exquisitely demonstrated a role of Crry in preventing tubular injury by inducing tubulointerstitial damage utilizing renal artery perfusion of anti-Crry antibody into normal rat kidneys [83]. This discrepancy may be explained by a predominant effect of anti-Crry administration on vascular Crry. As anti-Crry antibody could bind to the peritubular vasculature and alter endothelial function associated with increased vascular permeability [84], increased access of the complement components derived directly from the circulation may have caused tubulointerstitial injury in normal rats in their studies. Hatanaka et al also showed significant tubulointerstitial injury in anti-glomerular basement membrane (GBM) nephritis rats treated with anti-Crry [85]. Increased accessibility of anti-GBM antibodies to tubular basement membrane was demonstrated in their animals, which suggested that deranged vascular permeability by neutralization of Crry led to the tubulointerstitial damage in their studies.

Although antisense treatment of normal rats had no effect, activation of the complement cascade in tubules of PAN rats was more severe in the antisense-treated animals (Figs. 5 and 6). Histological analysis demonstrated that the more severe complement deposition was associated with profound tubulointerstitial damage in PAN rats treated with antisense ODNs (Fig. 7). These histological assessments were confirmed by staining for vimentin, a marker of renal tubular injury. Vimentin, a member of the family of intermediate filaments, is expressed in glomeruli and renal vasculature, but not in tubular cells, in normal rats [86]. Alterations seen in damaged tubules, regardless of etiology, include a neoexpression of vimentin [86, 87]. PAN rats treated with antisense ODNs showed more vimentin-positive tubules (Fig. 7). The amounts of proteinuria in antisense-treated animals and in control rats were not statistically different, implying that the differences in tubular complement deposition and tubulointerstitial damage were specific consequences of the decrease in Crry expression and consequent increased complement activation.

While PAN rats treated with antisense ODNs showed more severe tubulointerstitial damage than those treated sense ODNs, the two groups of animals did not show any difference in renal function despite distinct relationships between tubulointerstitial damage and impairment of kidney function established in previous human studies [1, 4–13]. This may be explained by the relatively short observation period in our studies (12 days) in contrast to the clinical studies demonstrating a significant correlation between tubulointerstitial injury and a loss of renal function during the follow-up of years or decades.

In conclusion, the decrease in Crry expression in proximal tubules induced by the antisense ODN approach was shown to induce significant activation of the complement cascade on the brush border of proximal tubules, leading to tubulointerstitial injury in proteinuric rats. Our studies confirm a pathogenic role for complement components contained in proteinuria leading to tubulointerstitial injury and, to our knowledge for the first time, document a protective role for a complement regulatory protein, Crry, in renal tubular cells.

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APPENDIX

Abbreviations used in this article are: ATS, antithymocyte serum; BUN, blood urea nitrogen; DAF, decay-accelerating factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GBM, glomerular basement membrane; GFR, glomerular filtration rate; HE, hematoxylin and eosin; LDH, lactate dehydrogenase; MCP, membrane cofactor protein; ODNs, oligodeoxynucleotides; PAN, puromycin aminonucleoside nephritis; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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