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C5b-9 regulates peritubular myofibroblast accumulation in experimental focal segmental glomerulosclerosis

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C5b-9 regulates peritubular myofibroblast accumulation in experimental focal segmental glomerulosclerosis.

Background. In human focal segmental glomerulosclerosis (FSGS), the tubulointerstitial deposition of the complement (C5b-9) membrane attack complex is correlated with interstitial myofibroblast accumulation and proteinuria. Here, we hypothesized that C5b-9 formation regulates renal myofibroblast accumulation in Adriamycin nephropathy.

Methods. Adriamycin nephropathy was induced in complement C6-sufficient (C6+) and C6-deficient (C6−) piebold viral glaxo (PVG) rats. Groups of animals ($N = 7$ to 8 each) were examined on days 21 and 42. A group of C6+ animals, injected with vehicle, served as the control group.

Results. C6+ and C6− rats with Adriamycin nephropathy had equivalent proteinuria. C5b-9 deposition was increased and present on the apical surface of proximal tubular epithelial cells (day 21 and 42) and peritubular region (day 42 only) in C6+ rats with Adriamycin nephropathy, and absent in C6− rats. Peritubular myofibroblast accumulation increased in a time-dependent manner in C6+ proteinuric rats (control 1.2 ± 0.4; Adriamycin nephropathy day 21 11.0 ± 0.7; Adriamycin nephropathy day 42 19.8 ± 1.7 cells per high power field). In C6− rats this increase was blunted by 87% and 56% on days 21 and 42, respectively ($P < 0.01$), and was associated with reduced interstitial extracellular matrix (ECM) deposition. Tubulointerstitial injury, tubular vimentin and interstitial monocyte accumulation were also reduced in C6− rats with Adriamycin nephropathy on day 21, but not at day 42. In contrast, the increase in periglomerular myofibroblast accumulation and glomerulosclerosis in Adriamycin nephropathy were not altered by C6 deficiency.

Conclusion. These data suggest that glomerular ultrafiltration of complement components and the intratubular formation of C5b-9 is a specific promotor of peritubular myofibroblast accumulation in FSGS.

Primary focal segmental glomerulosclerosis (FSGS) is a noninflammatory glomerulonephritis, characterized by persistent nephrotic syndrome, nonselective proteinuria, microscopic hematuria, hypertension, and renal impairment [1]. Irreversible injury to podocytes underlies the glomerular permselectivity loss, and in humans, the cause/s may include a circulating podocyte toxic permeability factor, congenital and acquired mutations in podocytes proteins, and/or viral infection [1]. Nephrotic-range proteinuria, chronic tubulointerstitial damage, and corticosteroid resistance are predictors of a poor renal prognosis in FSGS [1–3]. In such patients, currently available treatments are disappointing, and the clinical course is characterized by progressive deterioration to end-stage kidney failure in up to 50% of patients over a 10-year period [1].

Considerable evidence is accumulating that the glomerular ultrafiltration of serum-derived complement components and formation of the terminal membrane attack complex (C5b-9) in the tubular lumen is an important mechanism of tubulointerstitial damage in nephrotic glomerular diseases [4–9]. Clinical evidence to support this hypothesis in FSGS include (*1*) compared to other glomerular diseases, patients with FSGS have one of the highest rates of urinary C5b-9 excretion (15-fold higher than controls in one study), which is second only to diabetic nephropathy (increased more than 30-fold) [10, 11]; and (*2*) the tubulointerstitial deposition of C5b-9 is increased in patients with FSGS, particularly in those that do not respond to treatment with corticosteroids [12, 13].

Renal myofibroblast accumulation is also increased in patients with FSGS [13, 14]. Myofibroblasts are activated smooth muscle–like fibroblasts, phenotypically characterized by the filamentary expression of alpha-smooth muscle actin (α-SMA), vimentin, and desmin [15]. In tubulointerstitial damage, myofibroblasts could act as sentinel inflammatory cells [16], contribute extracellular matrix (ECM) deposition [15], and their fibrocontractile properties might theoretically lead to strangulation of tubules resulting in atubular glomeruli [17, 18]. Renal myofibroblast accumulation is also a predictor of progression in a variety of human glomerular diseases [19, 20].

Key words: complement, tubulointerstitial fibrosis, chronic kidney disease.

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These data therefore potentially implicate the myofibroblast as a significant cellular player in tubulointerstitial damage associated with FSGS. However, the mechanisms of myofibroblast accumulation in FSGS are poorly understood. It has been suggested that components of proteinuric urine could promote peritubular myofibroblast accumulation [20]. Interestingly, in patients with FSGS, the tubulointerstitial expression of C5b-9 is correlated with peritubular, but not periglomerular, α-SMA, accumulation [13].

Adriamycin nephropathy is a nonimmune mediated nephrotic rat model of FSGS [21]. The pathologic changes include early and prominent tubulointerstitial damage; podocyte foot effacement and detachment; and glomerular changes, which evolve from minimal change lesions into FSGS [21, 22]. Rodent Adriamycin nephropathy, similar to human FSGS, is resistant to corticosteroids [23] and shows variable renoprotection to angiotensin blockade [24]. In contrast to human FSGS, however, glomerular injury in rodent Adriamycin nephropathy is initiated by irreversible toxic injury to the glomerular capillary wall and podocytes by doxorubicin [21]. The aim of the present study was to examine the hypothesis that, in Adriamycin nephropathy, the increased glomerular filtration of complement components and formation of the membrane attack complex regulates renal myofibroblast accumulation. To test this hypothesis, Adriamycin nephropathy was induced in complement sufficient (C6+) and complement deficient (C6−) piebold-viral glaxo (PVG) rats, which are unable to form C5b-9 [25].

METHODS

Experimental animals

Forty two age and litter-matched adult male PVG rats (approximately 10 to 12 weeks in age; initial body weight 280 ± 1.5 g, mean ± SE) with ($N = 26$) or without C6 ($N = 16$) deficiency were obtained from the breeding colony at the University of Washington, Seattle, Washington. The original source for the breeding pairs with normal complement activity was Harlan-Sprague-Dawley (Cambridge, UK), and complement deficient was Bantin and Kingman Universal (Edmonds, Washington). Before the studies were commenced the complement status of each PVG rat was verified by measuring the hemolytic activity of serum by a standard CH50 assay as previously described [26]. All rats were housed in groups of two to three per cage in a temperature and light-controlled environment in accredited animal facility, with free access to food and water. The studies were performed in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory animals*. The Ethics Committee of the University of Washington, Seattle, approved protocols.

Induction of Adriamycin nephropathy

Adriamycin nephropathy was induced in an identical manner in C6+ ($N = 16$) and C6− ($N = 16$) male rat littermates by a single intravenous injection of doxorubicin hydrochloride (6.0 mg/kg) under anesthesia (single intraperitoneal injection of ketamine:xylazine 90:10 mg/kg) on day 0 [22]. A separate group of C6+ animals ($N = 10$) received a single injection of saline (vehicle) instead of doxorubicin. Groups of animals were sacrificed on day 21 and 42 ($N = 8$ each, C6+ and C6−, per Adriamycin nephropathy group and time point; $N = 5$ each, per control group and time point). One C6-sufficient animal with Adriamycin nephropathy (from the day 42 group) developed tense abdominal swelling due to ascites on day 34. Euthanasia was performed on day 35 due to respiratory distress. This rat was excluded from all subsequent analyses. The dose of Adriamycin chosen for this study was lower than previous studies (7.5 mg/kg [22]), because preliminary experiments showed that this dose caused more severe systemic Adriamycin toxicity (weight loss) in both C6+ and C6− PVG rats. The time points (days 21 and 42) were chosen according to previous studies using this model [22]. One day prior to tissue collection, animals were placed in metabolic cages for 17 hours to determine urine protein and creatinine excretion. At the time of sacrifice, animals were anesthetized with ketamine:xylazine, a midline laparotomy was performed, blood was collected from the inferior vena cava, and both kidneys were removed.

Renal function

Renal function and assessment of nephrotic syndrome was assessed by measuring urinary protein excretion, serum creatinine, urea, albumin, and cholesterol. Serum urea, creatinine, albumin, and cholesterol were measured by an autoanalyser (Hitachi B747) (PathCentre, QEII Medical Center, Nedlands, WA, Australia). Urinary protein excretion was measured by the sulfosalicylic acid method.

Renal histology and immunohistochemistry

Coronal sections of the kidney were immersion-fixed in methyl Carnoy's solution or 10% neutral-buffered formalin and embedded in paraffin. Light microscopy was performed on 4 μm sections of tissue stained with either periodic acid-Schiff (PAS) reagent (counterstained with hematoxylin) or Masson's trichrome.

Immunoperoxidase staining was performed as previously described [7, 8] using the following antibodies: a murine monoclonal IgG_2 antibody against α-SMA, a cytoskeletal marker of myofibroblastic differentiation (Sigma-Aldrich Chemical Co., St. Louis, MO, USA); a rabbit polyclonal antibody against rat fibronectin

(Chemicon, Temecula, CA, USA); a murine monoclonal IgG antibody V9 against vimentin (Dako, Carpinteria, CA, USA); and a murine monoclonal IgG_1 antibody against ED-1, a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (Bioproducts for Science, Inc., Indianapolis, IN, USA). Horseradish peroxidase-conjugated avidin D (Vector Laboratories, Burlingame, CA, USA) was used after all biotinylated secondary antibodies at room temperature for 20 minutes. The chromogen was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with methyl green. As a negative control, the primary antibody was omitted or substituted with preimmune rabbit, mouse or goat serum, corresponding to the species of the primary antibody.

Tissue for immunofluorescence was embedded in 22-oxacalcitriol (OCT) compound (Lab-Tek Products, Miles Laboratories, Naperville, IL, USA) and snap-frozen in liquid nitrogen. Rat C3 was detected with fluorescein isothiocyanate (FITC)-conjugated goat antirat C3 (ICN Pharmaceuticals, Irvine, CA, USA). The presence of rat C5b-9 was determined using biotinylated antirat C5b-9 monoclonal antibody 2A1 followed by FITC-streptavidin [8, 9, 22].

Quantitation of renal histology and immunohistochemistry

For quantitation of cells (α-SMA, ED-1), the number of nuclei with DAB-positive cytoplasmic staining was determined. In the cortical interstitial space, the number of DAB-positive cells was counted in 20 nonoverlapping random fields (at a magnification of ×200), using a 0.5 mm^2 graticule fitted in the eyepiece of a microscope (BX51) (Olympus Australia, Welshpool, WA, Australia). The mean number per animal was calculated and expressed per mm^2 area. Similarily, in the glomerulus, the number of periglomerular α-SMA–positive cells was counted in at least 20 glomerular cross-sections per animal (magnification ×400).

For quantitation of tubular vimentin, the number of positive tubular cross-sections was counted in 20 nonoverlapping random fields (magnification of ×200 using a 0.5 mm^2 graticule). In order for tubules to be counted positive (and assigned a value of 1.0), at least one tubular epithelial cell in the tubular cross-section had to be DAB-positive. The mean number per animal was calculated.

Interstitial fibronectin was assessed by computer-assisted image analysis. For each animal, images were taken of 20 nonoverlapping random fields of the cortical interstitium not containing glomeruli (magnification of ×400) using a digital camera (Olympus DP11, 2MP, Olympus Australia, Welshpool, WA, Australia). The JPEG images were downloaded to a desktop computer, and the percent area occupied by interstitial fibronectin was calculated using image-analysis software (Optimas 6.51, Media Cybernetics, Silver Spring, MD, USA).

Tubulointerstitial injury (defined as tubular atrophy, dilatation, thickening of the basement membrane) and interstitial fibrosis (blue stain in Trichome-stained sections) by semiquantitative analysis [8, 9]. Thirty cortical fields from each animal were examined at ×200 magnification using a graticule (0.5 mm^2) and graded according to a scale of 0 to 4: 0, no tubulointerstitial injury or absent interstitial collagen deposition; 1, <25% of the tubulointerstitium injured or minimal deposition of interstitial collagen (<25%); 2, 25% to 50% of the tubulointerstitium injured or mild deposition of interstitial collagen (25% to 50%); 3, 51% to 75% of the tubulointerstitium injured or mild deposition of interstitial collagen (51% to 75%); and 4, 76% to 100% of the tubulointerstitium injured or diffuse deposition of interstitial collagen (>75%).

Statistical analysis

Data are expressed as mean ± SE. Comparisons between experimental groups were performed using the independent *t* test for parametric data, and Kruskal-Wallis test for nonparametric data. Tests to determine the distribution of data and all other calculations were performed using statistical software (JMP version 4.04) (SAS Institute, Carey, NC, USA). A $P < 0.05$ value was defined as being statistically significant.

RESULTS

Tubulointerstitial C5b-9 is increased in Adriamycin nephropathy

In control animals, C5b-9 immunoreactivity was rarely present, and localized as focal peritubular deposits (Fig. 1A and D). In C6+ rats with Adriamycin nephropathy, C5b-9 staining was increased. On day 21, C5b-9 was predominantly present in the lumen and apical surfaces of proximal tubular epithelial cells, similar to that observed in rats with puromycin aminonucleoside nephrosis (PAN) (Fig. 1B) [7]. In contrast, by day 42, C5b-9 staining was also increased in the peritubular region of the tubulointerstitium in nephrotic C6+ rats (Fig. 1E). Glomerular C5b-9 staining was absent in C6+ rats with Adriamycin nephropathy. Renal C5b-9 staining was completely absent in C6− animals (Fig. 1C and E).

C3 staining was present in a peritubular distribution in control animals and this was increased in rats with Adriamycin nephropathy (Fig. 1G). In addition, in Adriamycin nephropathy C3 was also detected in the lumen, apical surfaces and cytoplasm of tubular epithelial cells in C6+ rats with Adriamycin nephropathy. Tubulointerstitial C3 staining was similar in magnitude and distribution in both the C6− or C6+ groups at day 21 (Fig. 1G and

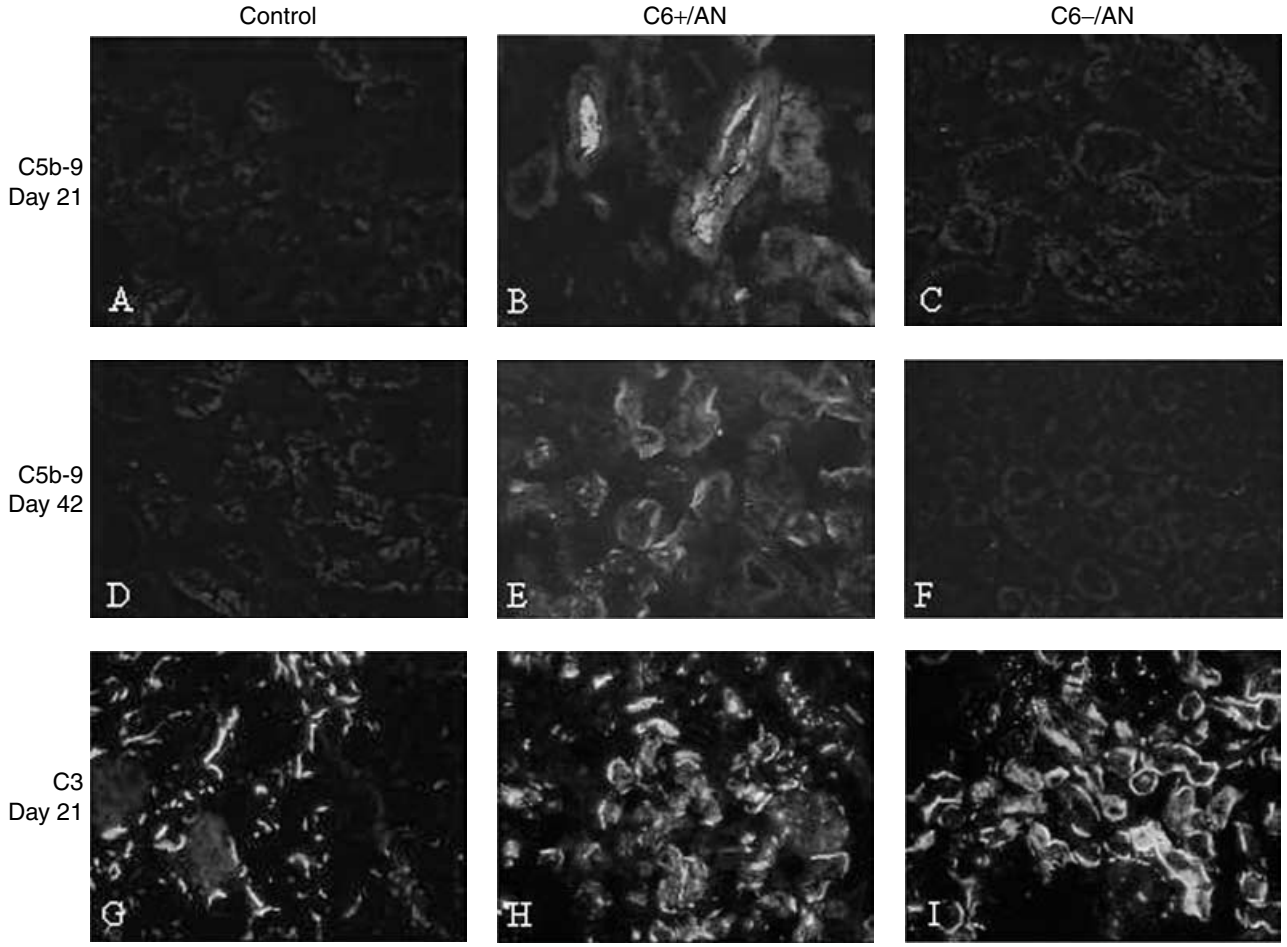

Fig. 1. Immunofluorescence microscopy of C5b-9 and C3 in the renal cortex. (*A* to C) Day 21 (C5b-9). (*D* to *F*) Day 42 (C5b-9). (*G* to *I*) Day 21 (C3). (A, D, and G) Saline-injected control C6+ animals. (B, E, and H) C6+ animals with Adriamycin nephropathy (AN). (C, F, and I) C6− animals with Adriamycin nephropathy (magnification ×400 for C5b-9; ×200 for C3).

H) and 42. Glomerular C3 staining was not detected in either group with Adriamycin nephropathy.

Body weight and kidney weight are similar in C6− and C6+ rats

Rats with Adriamycin nephropathy gained less body weight (Table 1), but no differences between the C6+ and C6− Adriamycin nephropathy groups, at any time point, were observed. Kidney weight increased in groups with Adriamycin nephropathy compared to the control group, but was not altered by C6-deficiency.

Proteinuria, renal function, and markers of nephrotic syndrome are similar in C6− and C6+ rats

In Adriamycin nephropathy, proteinuria increased and was 20-fold greater than that of control (vehicle-injected) animals (Fig. 2). The proteinuria peaked on day 21 and remained elevated on day 42 (Fig. 2). Levels of proteinuria were the same in both C6+ and C6− groups with Adriamycin nephropathy on both day 21 and day 42. Renal function, as assessed by elevation in the serum urea and creatinine, deteriorated in rats with Adriamycin nephropathy (Table 1). The absence of C6 did not alter the decline in renal function or development of biochemical markers of nephrotic syndrome (hypoalbuminemia, hypercholesterolemia) at any time point (Table 1).

Peritubular myofibroblast accumulation is attenuated in C6− rats on days 21 and 42

In control animals, α-SMA was strongly expressed in vascular smooth muscle cells of arteries, arterioles and smaller capillaries (Fig. 3A and D). In C6+ rats with Adriamycin nephropathy, the number of peritubular cells positive for α-SMA increased in a time-dependent manner (Figs. 3 and 4). Typically, the vast majority of these cells were elongated and located in a peritubular distribution, with their cellular processes almost surrounding the cross-section of a tubule. Round interstitial cells without elongated processes were also present, but much less common. In C6− rats with Adriamycin nephropathy,

Table 1. Body weight (BW), kidney weight (KW), and renal function in the experimental groups

Group	Initial BW	Final BW *g*	KW *g*	KW/BW *g/100g*	PUr *mmol/L*	PCr *μmol/L*	Palb *g/dL*	PChol *mmol/L*
Day 21								
Control	282 ± 1	290 ± 2	0.87 ± 0.01	0.30 ± 0.003	7.3 ± 0.2	37.8 ± 1.5	36.1 ± 0.7	2.2 ± 0.1
C6+	281 ± 2	260 ± 3[a]	1.12 ± 0.04[a]	0.43 ± 0.02[a]	11.9 ± 1.0[a]	70.9 ± 6.8[a]	14.4 ± 0.2[a]	13.7 ± 0.5[a]
C6−	280 ± 2	271 ± 2[a]	1.06 ± 0.03[a]	0.39 ± 0.01[a]	11.8 ± 0.6[a]	76.3 ± 9.7[a]	15.0 ± 0.5[a]	14.9 ± 0.7[a]
Day 42								
Control	284 ± 2	310 ± 3	0.87 ± 0.004	0.28 ± 0.003	6.9 ± 0.3	39 ± 2.1	35.7 ± 1.1	2.0 ± 0.1
C6+	281 ± 3	243 ± 6[a,b]	1.05 ± 0.05[a,b]	0.43 ± 0.02[a,b]	17.9 ± 1.9[a,b]	112.2 ± 5.6[a,b]	16.7 ± 0.4[a]	16.1 ± 1.4[a]
C6−	282 ± 2	237 ± 5[a,b]	1.07 ± 0.04[a,b]	0.45 ± 0.01[a,b]	20.9 ± 1.2[a,b]	97.8 ± 8.5[a,b]	15.4 ± 0.3[a]	17.3 ± 0.8[a]

Abbreviations are: SUr, plasma urea; PCr, plasma creatinine; Palb, plasma albumin; Pchol, plasma cholesterol. Data expressed as mean ± SE.
[a] $P < 0.05$ compared to control group.
[b] $P < 0.05$ compared to day 21 group.

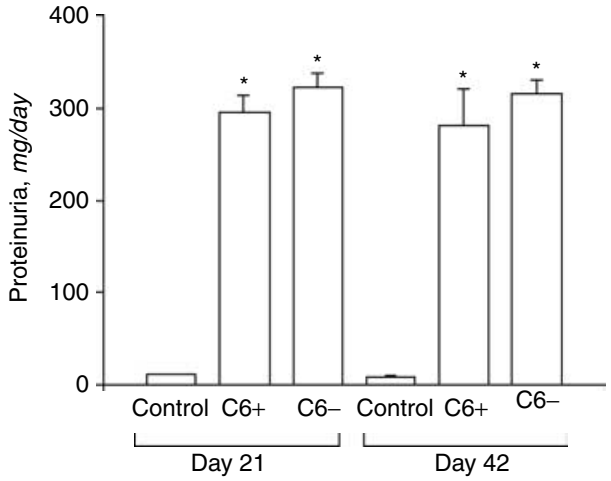

Fig. 2. Proteinuria in the experimental groups. Data expressed as mean ± SE. *$P < 0.01$ compared to control.

peritubular α-SMA accumulation was reduced (Figs. 3 and 4). This was most dramatic on day 21 when almost no α-SMA–positive cells were present in the interstitium, and partial on day 42.

Periglomerular myofibroblast accumulation is not altered in C6− rats at any time point

In control animals, there were no α-SMA–positive cells around glomeruli (Fig. 5A and D). In Adriamycin nephropathy, there was a dramatic increase in α-SMA–positive cells around glomeruli (Figs. 4 and 5). The intensity of cytosolic staining and number of cells was more prominent on day 42. However, there was no difference in number of periglomerular α-SMA–positive cells between the C6+ and C6− groups at either day 21 or 42 (Figs. 4 and 5).

Interstitial ECM deposition is attenuated in C6− rats on days 21 and 42

We next assessed whether the changes in myofibroblast accumulation were associated with alterations extracellular matrix in the interstitium [27, 28]. By Trichome staining, interstitial fibrosis increased in a time-dependant manner in Adriamycin nephropathy (Fig. 6). In C6-deficient animals, interstitial fibrosis was reduced on day 21 ($P < 0.01$) and day 42 compared to C6-sufficient ($P < 0.001$) (Fig. 6). Similarly, interstitial fibronectin deposition was also reduced in C6− rats with Adriamycin nephropathy, both on day 21 and day 42 (Fig. 6).

Markers of tubular injury and dedifferentiation are reduced in C6− rats on day 21 only

We next asked the question as to whether the decrease in myofibroblast accumulation in C6-deficient rats was due to a reduction in tubular epithelial cell injury [28, 29]. By semiquantitative analysis, tubulointerstitial injury was attenuated in C6− rats with Adriamycin nephropathy on day 21 ($P < 0.05$) but not at day 42 (Figs. 7 and 8A). Similarly, the number of tubules positive for vimentin was reduced at day 21 ($P < 0.01$) but not at day 42 (Fig. 8B). Therefore, attenuation of tubular injury did not explain the sustained reduction in myofibroblast accumulation in C6-deficient rats on day 42.

Interstitial monocyte accumulation is reduced in C6− rats on day 21 only

Because interstitial myofibroblast accumulation could be mediated by paracrine effects of macrophage [30], we examined whether ED-1 cell accumulation altered. Interstitial monocyte accumulation was increased in Adriamycin nephropathy ($P < 0.01$). Interstitial ED-1 accumulation was partly reduced on day 21 in C6− rats with Adriamycin nephropathy ($P < 0.05$), but this reduction was not sustained to day 42 (Fig. 8C).

Glomerulosclerosis and glomerular monocyte accumulation are not altered in C6− rats

There was a mild increase in glomerulosclerosis in rats with Adriamycin nephropathy. However, the absence of C6 did not alter the progression of glomerulosclerosis

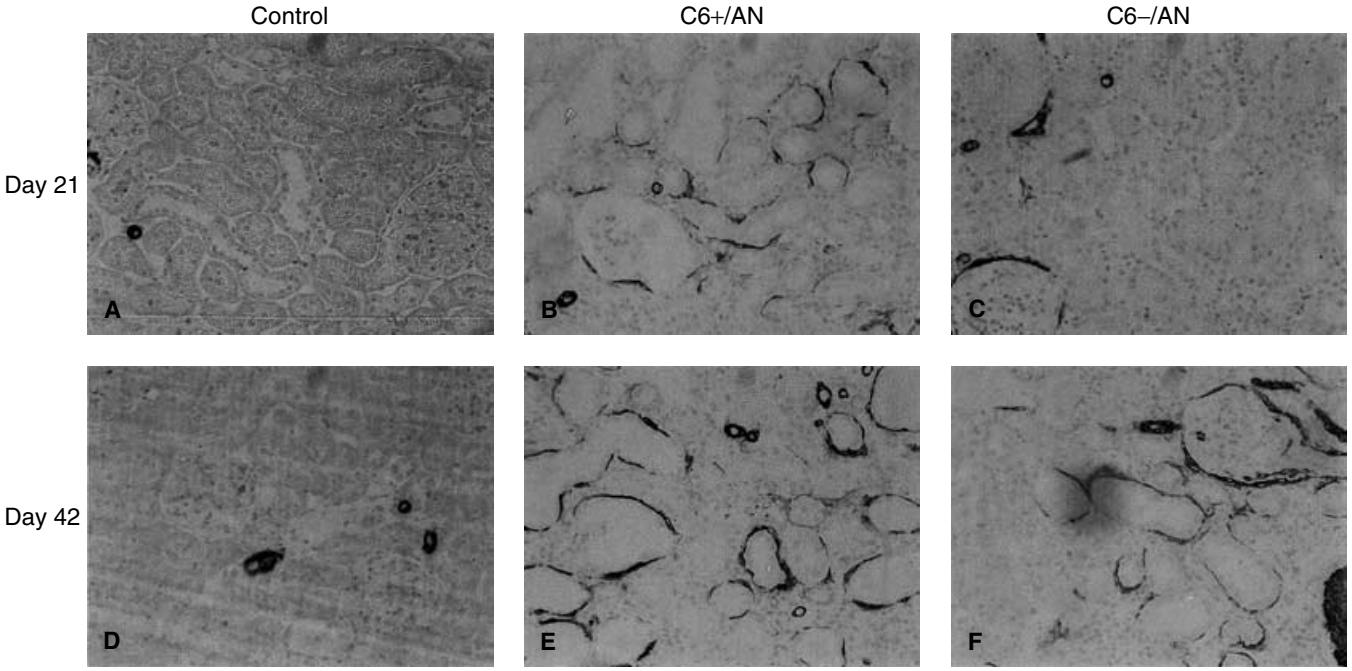

Fig. 3. Peritubular myofibroblast accumulation as determined by α-smooth muscle actin (α-SMA) immunohistochemistry. (*A* and *D*) Saline-injected control animals. (*B* and *E*) C6+ animals with Adriamycin nephropathy (AN). (*C* and *F*) C6− animals with Adriamycin nephropathy (magnification ×200).

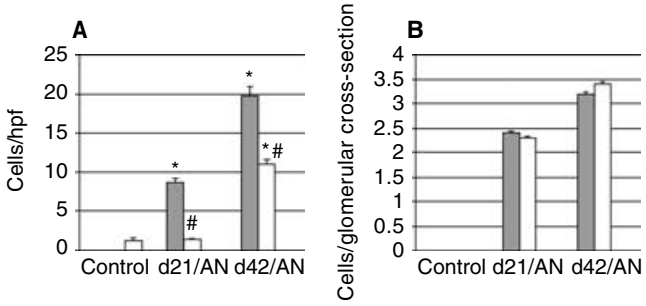

Fig. 4. Quantitation of peritubular (*A*) and periglomerular (*B*) α-smooth muscle actin (α-SMA)–positive cells. In the experimental groups on day 21 (d21) and day 42 (d42). Data expressed as mean + SE. $^{*}P < 0.01$ compared to control; #$P < 0.01$ compared to C6+/Adriamycin nephropathy (AN). Grey bars are C6+, white bars are C6−.

(Fig. 9A to C) or glomerular ED-1 cell accumulation (Fig. 9D to F).

DISCUSSION

The results of this paper show that the tubulointerstitial assembly of complement membrane attack complex, from serum-derived components in proteinuric urine, leads to the accumulation of myofibroblasts in the peritubular space, in Adriamycin nephropathy. The main findings were (*1*) C5b-9 formation was increased in the lumen and peritubular regions in kidney from rats with Adriamycin nephropathy; (*2*) the absence of tubulointerstitial C5b-9 formation in C6-deficient rats with Adriamycin nephropathy dramatically reduced the accumulation of α-SMA–positive peritubular cells; (*3*) renal interstitial fibrosis and fibronectin deposition (an ECM protein product of myofibroblasts) were reduced in C6-deficient rats with Adriamycin nephropathy; and (*4*) remarkably, the effects of C5b-9 on myofibroblasts were highly compartment-specific, because periglomerular accumulation of myofibroblast was not altered by C6-deficiency. Another important finding of this study was that tubular injury and activation, and interstitial monocyte infiltration were reduced in early, but not in the late stages of Adriamycin nephropathy.

Peritubular and periglomerular myofibroblast accumulation is increased in a variety of human and experimental models of chronic renal disease, and predicts a poor prognosis [19, 20]. In humans with primary FSGS, peritubular myofibroblast accumulation is also increased [13, 14] and correlated with the tubulointerstitial formation of C5b-9 [13]. In the remnant kidney model, protein overload of proximal tubular epithelial cells (in particular IgG) was associated with peritubular α-SMA accumulation [20]. Furthermore, reduction of proteinuria (and IgG accumulation in tubular cells) with an angiontensin-converting enzyme (ACE) inhibitor suppressed peritubular α-SMA accumulation [20]. The current study provides the first in vivo evidence directly demonstrating that C5b-9 is one of the components in proteinuric urine that is important in regulating peritubular myofibroblast accumulation in chronic proteinuric renal disease.

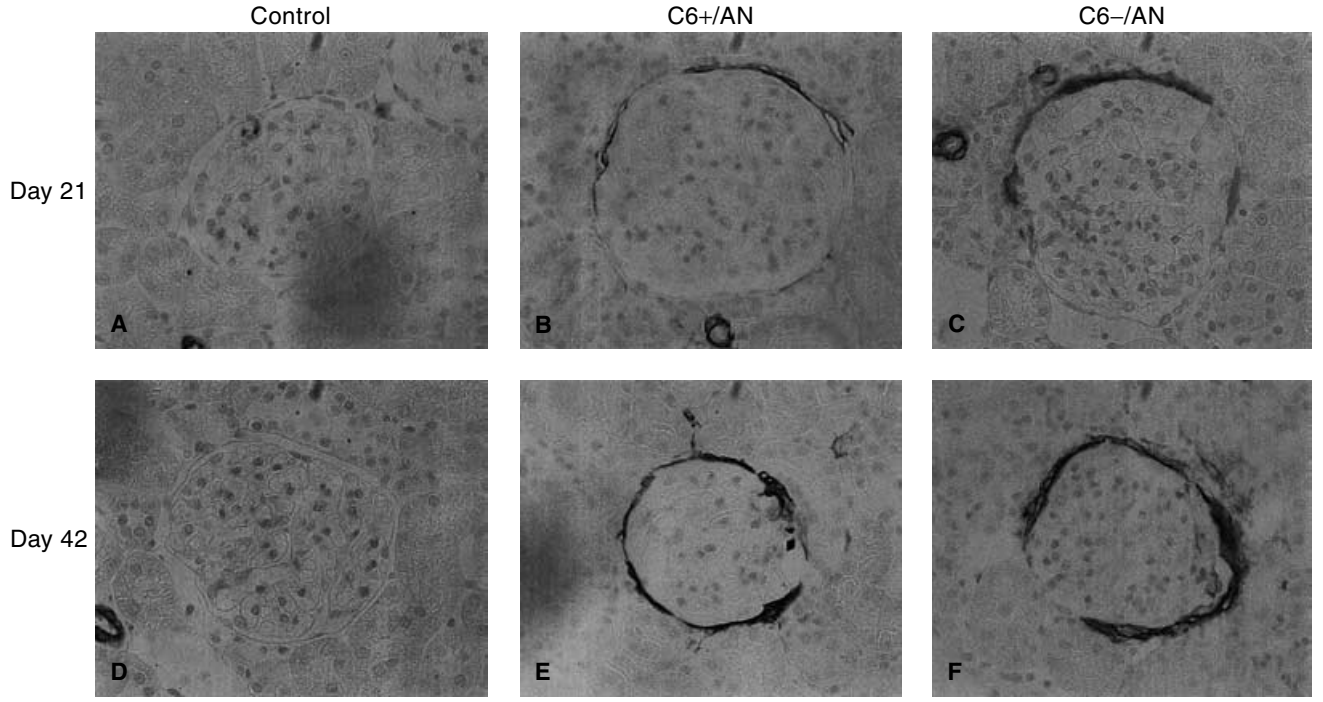

Fig. 5. Periglomerular myofibroblast accumulation. (*A* and *D*) Saline-injected control animals. (*B* and *E*) C6+ animals with Adriamycin nephropathy (AN). (*C* and *F*) C6− animals with Adriamycin nephropathy (magnification ×400).

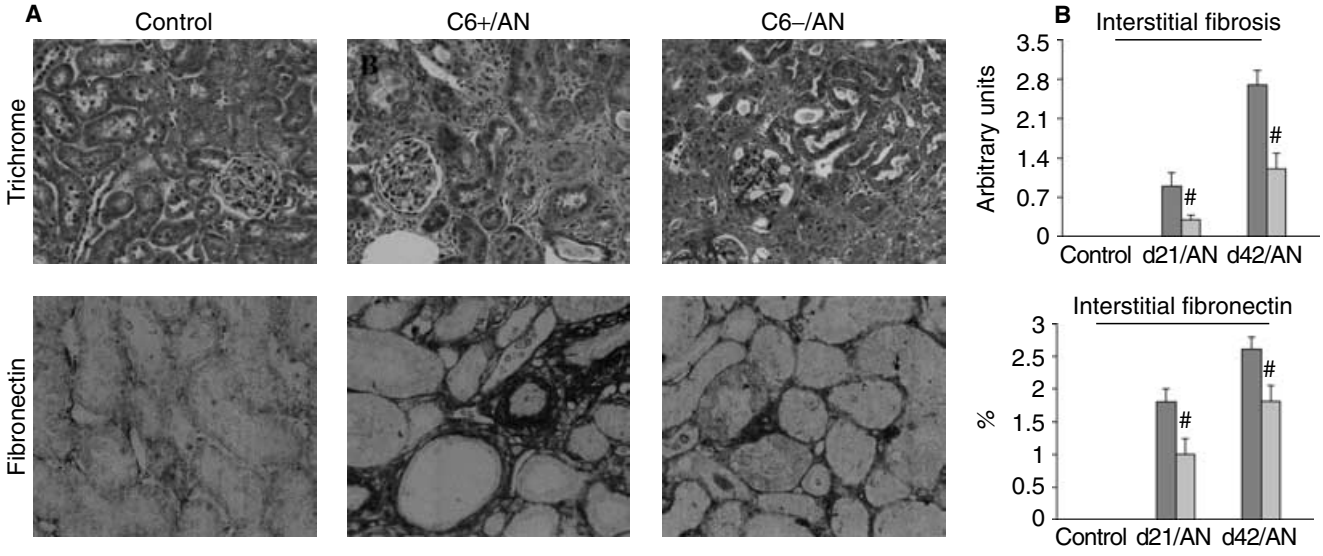

Fig. 6. (*A*) Interstitial fibrosis in Trichrome-stained sections (×200) and interstitial fibronectin deposition (×400) in the renal cortex on day 42. (*B*) Quantitative data for interstitial fibrosis and interstitial fibronectin deposition. Data expressed as mean + SE, $^{\#}P < 0.01$ compared to C6+/Adriamycin nephropathy (AN). Grey bars are C6+, white bars are C6−.

The origin of myofibroblasts in Adriamycin nephropathy and human FSGS remains uncertain, and could include differentiation from fibrogenitor stem cells or circulating precursor cells [15]; proliferation and increased survival of myofibroblasts [31]; and/or transdifferentiation from pericytes or proximal tubular epithelial cells [20, 32]. In Adriamycin nephropathy, at least four mechanisms could be involved in C5b-9–induced peritubular myofibroblast accumulation: (1) differentiation of local fibrogenitor cells due to the paracrine effects of fibrogenic growth factors [interleukin (IL)-1, transforming growth factor-α (TNF-α), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-β1 (TGF-β1)] released from

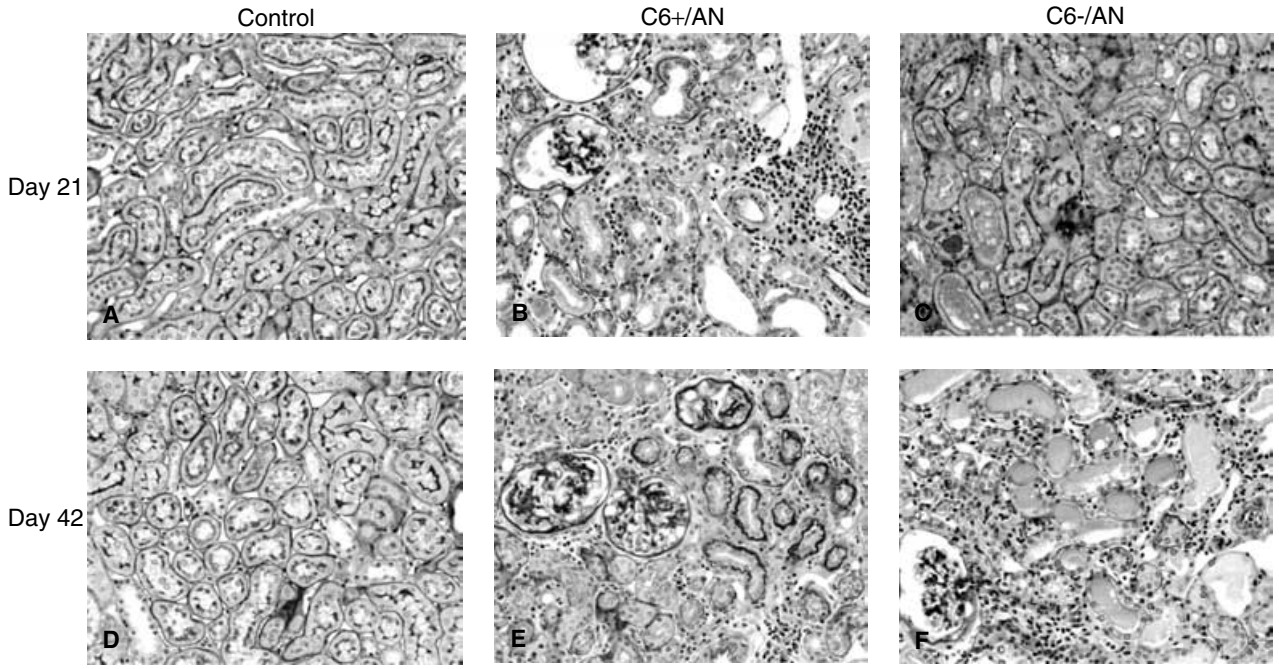

Fig. 7. Tubulointerstitial injury in periodic acid-Schiff (PAS)-stained sections. (*A* and *D*) Saline-injected control animals. (*B* and *E*) C6+ rats with Adriamycin nephropathy (AN). (*C* and *F*) C6− rats with Adriamycin nephropathy (magnification ×200).

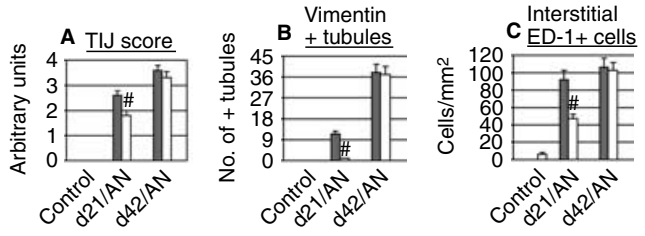

Fig. 8. Quantitation of cortical tubulointerstitial injury, vimentin-positive tubules, and interstitial endothelin-1 (ED-1)+ cell accumulation in the experimental groups. Data expressed as mean + SE. #$P <$ 0.01 compared to C6+/Adriamycin nephropathy (AN). Grey bars are C6+, white bars are C6−.

tubular epithelial cells exposed to C5b-9 [29, 33]. However, in coculture cell culture studies from our laboratory, the proliferation of normal human dermal fibroblasts was reduced when exposed to supernatants from human HK-2 cells (a proximal tubular epithelial cell line) stimulated with complement-sufficient serum compared to complement-deficient serum [abstract P125; Rangan GK, et al, *Nephrology* 7(Suppl):A74, 2002]. (*1*) These data suggest that complement does not increase the release of fibroblast mitogens by tubular cells; (*2*) differentiation of local fibrogenitor cells is due to the paracrine effects of macrophages, attracted because of chemokines released by C5b-9–activated tubular cells [15, 33]. However, in our study, monocyte accumulation and tubular epithelial cell injury was not altered on day 42, suggesting that this is only a partial explanation; (*3*) proliferation and activation of fibroblasts follows direct contact with C5b-9 in the interstitial space [34]. In our study, however, C5b-9 was only detected in the peritubular region on day 42, whereas myofibroblast accumulation was reduced prior to this time point; and (*4*) mesenchymal transition of tubular epithelial or endothelial cells into myofibroblasts is stimulated by C5b-9 [20, 32]. We postulate that all of these mechanisms probably have a role, to varying degrees, in FSGS and other nephrotic glomerular disorders, depending on the stage of the disease.

The current study extends previous data about the importance of C5b-9 as a mediator of tubular injury and interstitial inflammation [4–9]. In particular, recently Turnberg et al reported that tubulointerstitial damage was exacerbated in mice with Adriamycin nephropathy, that were deficient in CD59 (a complement regulatory protein that inhibits C5b-9) [abstract F-PO463; Turnberg et al, *J Am Soc Nephrol* 14:163A, 2003]. In previous studies we demonstrated that the absence of C6 attenuated tubulointerstitial damage in rats with PAN and the remnant kidney model [7, 8]. The present study further extends these findings in an irreversible and nonablative model of FSGS. However, an extremely important finding of the current study (which contrasts with our data in PAN and remnant kidney studies) was that tubular injury (as determined by semiquantitative scoring system and vimentin expression) was almost completely attenuated during the early time point of Adriamycin nephropathy (day 21), but not at the later time point (day 42). Similarly, interstitial monocyte accumulation was suppressed by nearly 50% (but still significantly

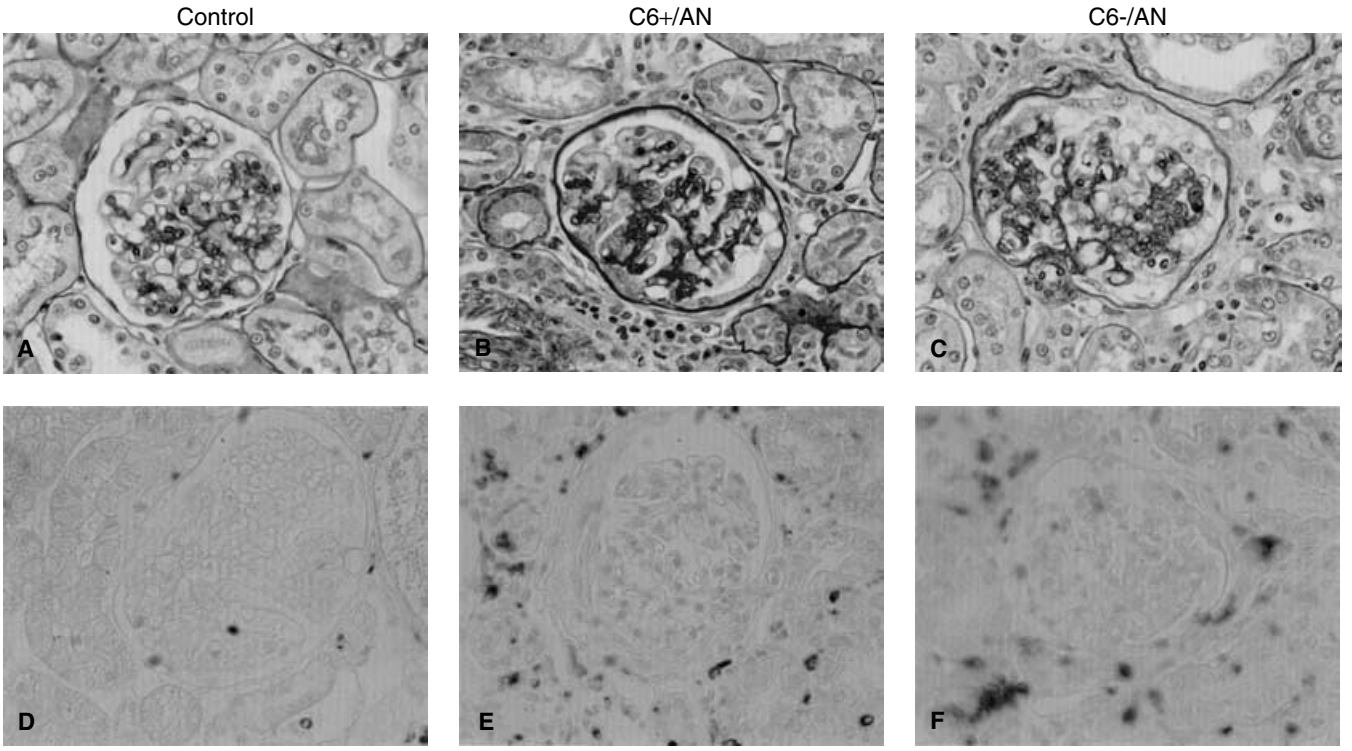

Fig. 9. Glomerulosclerosis in periodic acid-Schiff (PAS)-stained sections (*A* to *C*) and glomerular ED-1 accumulation (*D* to *F*) on day 42. AN is Adriamycin nephropathy (magnification ×200).

elevated compared to control animals) on day 21, but not on day 42. These pathologic findings, along with the persistence of proteinuria, could explain why C6-deficiency also did not alter the decline in renal function in Adriamycin nephropathy at any time point [35]. Moreover, the current data suggests that, at least in Adriamycin nephropathy, other mediators are also involved in causing tubulointerstitial injury. With regard to other components of the complement cascade, for example, the deficiency of C6 does not prevent activation such as C5a [36]. In that respect, Welch et al [37] reported that C5a is an important regulator of interstitial inflammatory cell accumulation in mice with chronic proteinuric renal disease.

An interesting observation of the current study was the presence of C5b-9 in the lumen of tubular epithelial cells (at day 21) and also peritubular regions (at day 42) in Adriamycin nephropathy. In previous studies of the PAN and remnant kidney models [7, 8], C5b-9 was restricted only to the lumen of tubular epithelial cells. The luminal formation of C5b-9 from filtration of complement components is thought to be due to at least three mechanisms: (*1*) C3-convertase activity of the proximal tubular epithelial cell brush border [38]; (*2*) amidation of C3 by ammonia [4]; and (*3*) absence of CD59 on the tubular lumen [39]. However, peritubular C5b-9 formation is not unexpected, and, in fact, consistent with pathologic studies of human renal diseases (including FSGS) [40, 41]. C5b-9 formation under these circumstances could be due to extravasation of complement components in an inflamed tissue microenvironment catalyzed by the C3-convertase activity of damaged parenchymal cells [42]. Another possibility is that peritubular C5b-9 could also arise from misdirected glomerular ultrafiltrate as suggested by Kriz [17], although this might be a relative late mechanism in Adriamycin nephropathy (occurring at 16 weeks) [18]. At least theoretically, peritubular C5b-9 could have direct sublytic effects on interstitial cells and thereby contribute to tubulointerstitial damage [34, 43]. However, in separate studies from our laboratory, tubulointerstitial damage in rats with ureteric obstruction and cyclosporin nephropathy (in which tubulointerstitial, but not luminal C5b-9, deposition is present due to the absence of proteinuria) was not altered by C6-deficiency [abstract P126, Rangan GK, et al, *Nephrology*, 7(Suppl):A74, 2002]. These data suggest that peritubular C5b-9 is not essential for tubulointerstitial damage, or that other additional mechanisms are involved in renal diseases characterized by proteinuria.

In a previous study in Adriamycin nephropathy, tubulointerstitial damage was not altered in rats with a congenital deficiency of albumin [44]. It is possible that the persistence of other components of proteinuric urine [45], or lipid moieties bound to plasma proteins in the analbuminemic rats [46], ensured that tubulointerstitial lesions developed in these animals. Indeed, the data from the present study directly supports that complement (and C5b-9) is one of the principal noxious filtered proteins.

The relative significance of other noxious proteins, such as transferrin and immunoglobulins, requires further investigation.

Although the pathologic lesions in Adriamycin nephropathy resemble human FSGS, the limitations of this model are that the etiology of glomerular injury is different to human disease, and the extent to which doxorubicin per se contributes to tubulointerstitial lesions in Adriamycin nephropathy is unknown [21]. In the current study both the C6-sufficient and deficient groups were matched by body weight and age, and all animals received an identical dose of Adriamycin. Moreover, glomerular injury, proteinuria and markers of nephrotic syndrome (hyperlipidemia and hypoalbuminemia) were similar the C6+ and C6− groups. Therefore, differences in dose of Adriamycin, degree of glomerular injury or systemic effects of nephrotic syndrome could not explain the striking reduction in peritubular myofibroblast accumulation in C6-deficient group.

CONCLUSION

The present study provides further evidence for complement and C5b-9 as a pathogen for tubulointerstitial damage associated with nephrotic glomerular diseases, such as FSGS. This together with other studies [4–13] gives substantial preclinical support for the testing and development of an inhibitor of C5b-9 formation in chronic proteinuric renal disease, preferably one that is orally active, cost-effective and renal-specific. Clearly, such a therapy could "complement" existing renoprotective agents, and hopefully make treatment of steroid-resistant FSGS far more gratifying for the clinical nephrologist in the future.

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