

Basic Study

C5b-9 does not mediate tubulointerstitial injury in experimental acute glomerular disease characterized by selective proteinuria

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Abstract

AIM: To determine whether complement membrane attack complex (C5b-9) has a pathogenic role in tubulointerstitial injury in a renal disease model characterized by acute highly selective proteinuria.

METHODS: Protein-overload nephropathy (PON) was induced in adult female Piebald-Viral-Glaxo rats with or without complement C6 deficiency (C6⁻ and C6⁺) by daily intraperitoneal injections of bovine serum albumin (BSA, 2 g/d), and examined on days 2, 4 and 8.

RESULTS: Groups with PON developed equivalent levels of heavy proteinuria within 24 h of BSA injection. In C6⁺ rats with PON, the tubulointerstitial expression of C5b-9 was increased and localized predominantly to the basolateral surface of tubular epithelial cells (TECs), whereas it was undetectable in C6⁻ animals. TEC proliferation (as assessed by the number of BrdU+

cells) increased by more than 50-fold in PON, peaking on day 2 and declining on days 4 to 8. There was a trend for a reduction in the number of BrdU+ TECs on day 4 in the C6⁻ PON group ($P = 0.10$ compared to C6⁺) but not at any other time-point. Kidney enlargement, TEC apoptosis (TUNEL⁺ cells) and markers of tubular injury (tubule dilatation, loss of TEC height, protein cast formation) were not altered by C6 deficiency in PON. Interstitial monocyte (ED-1+ cell) accumulation was partially reduced in C6⁻ animals with PON on day 4 ($P = 0.01$) but there was no change in myofibroblast accumulation.

CONCLUSION: These data suggest that C5b-9 does not mediate tubulointerstitial injury in acute glomerular diseases characterized by selective proteinuria.

Key words: Apoptosis; Proliferation; Tubulointerstitial; Proteinuria; C5b-9; Complement; Rats

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Core tip: The intra-renal assembly of the complement membrane attack complex (C5b-9) in the tubular lumen may be one of the principal mediators of chronic tubulointerstitial damage in nephrotic glomerular disease. This study shows that in an acute glomerular disease model (protein overload nephropathy) characterized by the rapid onset of highly selective proteinuria, C5b-9 does not mediate early tubulointerstitial injury. This may be due to the low luminal formation of C5b-9 in this model, and suggests that other factors, such as the filtration of albumin, growth factors and/or microtubular protein-cast obstruction, are more important in the pathogenesis of tubulointerstitial injury under these circumstances.

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INTRODUCTION

In humans with chronic glomerular disease, the degree of tubular atrophy and interstitial disease are the strongest histological parameters which correlate with renal function and predict progression^[1]. Irreversible damage to the glomerular capillary wall and ultrafiltration of serum-derived proteins into the tubular lumen is one of the non-immunologic mechanisms that evokes secondary tubulointerstitial disease, a process that is independent of the original inciter of glomerular injury^[2,3]. Clinical^[4-11] and experimental^[12-21] evidence suggests that the abnormal presence of serum-derived complement components in the tubular lumen

during proteinuric states, leads to the assembly of the complement membrane attack complex (C5b-9) (via the alternative pathway) on the apical brush border of tubular epithelial cells (TECs), and that this is an important factor in the causation of tubulointerstitial damage in proteinuric renal diseases^[22-26]. Under these conditions, the binding of serum-derived properdin (the only known positive regulator of the alternative pathway) to tubular heparin-sulfate is a pivotal facilitator of C5b-9 formation on the apical surface of the tubular lumen^[27-30].

In previous studies, the progression of tubulointerstitial damage has been compared in various models of non-immune mediated chronic kidney disease using rats unable to generate C5b-9 (due to the genetic absence of the C6 complement component)^[16,17,31,32]. In chronic proteinuric models (puromycin aminonucleoside, PAN; remnant kidney, RK; adriamycin nephropathy, AN) C5b-9 was localised to the tubular lumen and brush border of TECs^[16,17,32], and tubulointerstitial injury was attenuated in C6 deficient rats compared to the C6 replete group, despite equivalent proteinuria and renal function. Conversely, in non-proteinuric models, only peritubular (but not luminal) C5b-9 formation occurred, and C6 deficiency did not alter the progression of tubulointerstitial damage under these circumstances^[31]. Thus, these data emphasise that intraluminal C5b-9 formation may be an important determinant of whether it has a pathological role in chronic kidney diseases.

In humans, the urinary excretion of C5b-9 (a marker of intraluminal complement formation) is dependent on the type of glomerular pathology, being highest in diseases characterized by non-selective proteinuria (such as focal segmental glomerulosclerosis, diabetic nephropathy and membranous nephropathy) and absent in minimal change disease (a disease characterized by highly selective proteinuria)^[6,33]. These observations suggest that proteinuria *per se* is not a prerequisite for intraluminal C5b-9 formation, and that it may not mediate tubulointerstitial damage in glomerular disease (as in minimal change disease) characterized by highly selective proteinuria^[34,35]. To test this hypothesis in the preclinical setting, protein-overload nephropathy (PON) was induced in rats deficient or sufficient in complement C6. In contrast to PAN and AN, the proteinuria in PON is almost immediate in onset and highly selective in composition^[36]. During the first week, PON is characterised by marked renal enlargement^[37], TEC proliferation^[38] and mild interstitial inflammation^[14].

MATERIALS AND METHODS

Animals

Female Piebald-Viral Glaxo (PVG) rats with ($n = 28$) or without ($n = 25$) C6 deficiency were obtained from the breeding colony at the University of Washington, Seattle, WA, United States (Body weight 173 ± 3 g, mean \pm SEM)^[39]. The original source for the breeding pairs with normal complement activity was Harlan

Sprague-Dawley (Cambridge, United Kingdom) whereas for the C6 deficient animals it was Bantin and Kingman Universal (Edmonds, WA, United States)^[40]. Before the study, the haemolytic activity in serum from each rat was measured by a standard CH₅₀ assay^[39]. All rats were housed in groups of three to four per cage under standard laboratory conditions and allowed free access to commercial rat pellets and tap water. Experimental protocols were approved by the Animal Use Care Committee of Westmead Hospital (Protocol No. 135.02-08) and the Animal Use Committee at the University of Washington. The study was conducted in accordance with the Australian Code for the care and use of animals for scientific purposes and the National Institutes of Health Guide for the Use and Care of Laboratory animals.

Experimental model of PON

PON was induced in groups of animals by daily intraperitoneal injections of bovine serum albumin (2 g, BSA, A4503, Sigma-Aldrich, St Louis, United States), as previously described^[38], from day 1 until day 8 (i.e., total of eight consecutive injections) under ether anaesthesia, and groups of animals were sacrificed on days 2, 4 and 8. A separate group of control animals received saline only and were sacrificed at the same timepoints ($n = 2$ C6⁺, $n = 1$ C6⁻ per timepoint). Four animals with PON ($n = 2$ C6⁺ and $n = 2$ C6⁻) died during the study and were excluded from all subsequent analyses. Three were due to respiratory arrest from ether anaesthesia and another was euthanased on day 3 due to weight loss and physical signs of distress.

On the day prior to sacrifice, rats were placed in metabolic cages for 16 h to assess proteinuria. To assess the effects of C6 deficiency on TEC proliferation, 3 h prior to sacrifice, animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU, 50 mg/kg, Amersham Life Science). At the time of sacrifice, animals were anaesthetised by an intraperitoneal injection of ketamine: Xylazine, a mid-line laparotomy was performed, the inferior vena cava and aorta were transected and both kidneys were removed and weighed.

Renal function and proteinuria

Proteinuria was assessed by the sulfosalicylic acid method^[32]. Urinary creatinine, serum creatinine, urea, albumin and total protein were assessed by the Institute of Clinical Pathology and Medical Research, Westmead Hospital using an auto-analyzer^[32].

Histology

Coronal sections of the kidney were immersion-fixed in methyl Carnoy's solution or neutral-buffered formalin and embedded in paraffin^[32]. Arbitrary coronal sections, 4 μ m in thickness, were stained with periodic acid-schiff (PAS). Tissue for immunofluorescence was embedded in OCT compound (Lab-Tek products, Miles Laboratories, Naperville, IL, United States) and snap-frozen in liquid nitrogen^[32].

C5b-9 immunohistochemistry

The presence of rat C5b-9 was determined using biotinylated anti-rat C5b-9 monoclonal antibody 2A1 followed by fluorescein isothiocyanate streptavidin, as previously described^[16,17,31,32].

Assessment of TEC proliferation

TEC proliferation was assessed by immunohistochemistry using antibodies against proliferating cell nuclear antigen (PCNA) and BrdU. Tissue sections were deparaffinized with Histoclear^R (National Diagnostics, Atlanta, GE, United States) and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min, followed by incubation with a blocking agent (Background Buster, Accurate Chemical and Scientific Corporation, Westbury, NY, United States). The kidney sections were then incubated with either of the following primary and secondary antibodies: A mouse antibody reactive against anti-BrdU (Amersham Biosciences, United Kingdom), followed by a biotinylated rabbit anti-mouse IgG_{2a} antibody. Immunoreactivity of the tissue sections was visualized with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, United States) and DAB. As a negative control for non-specific immunoreactivity, pre-immune serum (from the same animal species as the primary antibody) was substituted in place of the primary antibody, with each staining procedure. The slides were counterstained with 2% methyl-green or PAS. In some slides, double immunohistochemistry for proximal TEC brush border using anti-rabbit Fx1A and PCNA was performed, counterstained with PAS.

Assessment of TEC apoptosis

Apoptosis was evaluated by the *in situ* cell death detection terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) method using a commercial kit (Roche Diagnostics, Sydney, Australia). The TUNEL method was performed on formalin-fixed slides according to the manufacturer's instruction. Permeabilisation was achieved with proteinase K (20 g/mL) treatment. TUNEL positive cells were visualised with DAB. Positive and negative controls were prepared as recommended by the manufacturer. TUNEL positive cells were defined according to strict criteria as DAB positive cells with morphological features of apoptosis.

Assessment of tubular injury and interstitial inflammation

Tubular injury was assessed by immunohistochemistry for antibodies against vimentin (marker of TEC dedifferentiation), ED-1 (marker of monocytes and macrophages) and α -smooth muscle actin (SMA, marker of myofibroblasts), as previously described^[31,32].

Quantification of immunohistology

Random selection methods were used to determine the microscopic fields for evaluation. For PCNA, percentage area of positive staining in the renal cortex was

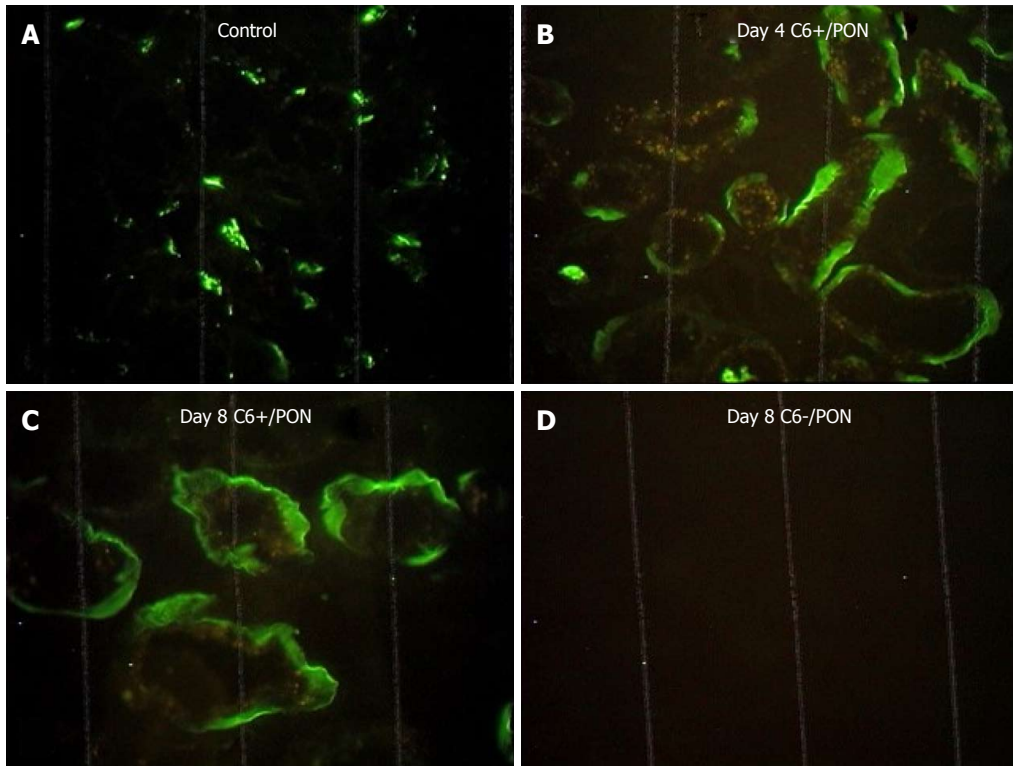


Figure 1 The tubulointerstitial deposition of C5b-9 is increased in C6⁺ rats with protein-overload nephropathy. Representative sections are shown. A: Animal injected with saline (control); B: C6⁺ animal with PON on day 4; C: C6⁺ animal with PON on day 8; D: C6⁻ animal with PON on day 8 ($\times 400$ magnification). Predominant basolateral deposition of C5b-9 was present in PON. PON: Protein-overload nephropathy.

assessed by quantitative image analysis in 10 cortical fields, as previously described, using Optimas Image analysis software^[32]. For BrdU, TUNEL and ED-1, the number of positive cells/nuclei were counted in 10 non-overlapping cortical fields ($\times 200$). In addition, previous studies in PON^[38] showed that TEC TUNEL positivity in this model increases from the outer cortex (subcapsular region, defined as Field 1) to the inner cortex/outer medulla (defined as Field 4), and therefore ten separate fields for each region was evaluated separately in each slide stained for TUNEL.

To determine mean tubule diameter and TEC height, images were digitized and viewed on a computer and analysed with public-domain image analysis software (Image J version 1.33, NIH software). To assess tubule diameter, the shortest cross-sectional diameter of a tubule was measured by line morphometry, as previously described^[31,32]. At least five non-overlapping but contiguous cortical fields ($\times 400$ magnification) and three tubules (the largest three) were assessed per field. The tubule cell height was the perpendicular length of a TEC.

Statistics analysis

The data were analyzed with JMP statistical software package (version 4.04, SAS institute, Carey, NC, United States). Data are expressed as mean \pm SE, median and interquartile range. Comparisons between control group and the complement sufficient group with PON were performed the Kruskal-Wallis test followed by

the Tukey-Kramer honest significance difference. To determine the effect of complement deficiency in PON, comparisons between C6⁺ and C6⁻ groups with PON at each timepoint were performed using the Wilcoxon test. A *P* value of less than 0.05 indicated statistical significance.

RESULTS

Tubulointerstitial deposition of C5b-9 is increased in C6⁺ rats with PON

By immunohistochemistry of the kidney, in control C6⁺ animals, occasional focal areas of C5b-9 were present in the peritubular region of the tubulointerstitium (Figure 1A). In C6 sufficient animals with PON, C5b-9 deposition was increased compared to control animals but localized to the basolateral membrane of TECs (Figure 1B). This pattern of C5b-9 immunoreactivity increased throughout the time-course, peaking on day 8 (Figure 1C). Only occasional and rare areas of C5b-9 could be detected on the luminal brush border in PON. C5b-9 was completely absent in C6⁻ deficient animals with PON (Figure 1D).

Serum protein and urinary protein excretion are similar in C6⁺ and C6⁻ rats with PON

Both total protein and albumin increased in the serum of C6⁺ rats with PON at all time-points (Table 1). This was paralleled by the development of marked proteinuria. Severe proteinuria was detected by urine

Table 1 Biochemical data in the experimental groups

Group	n	Serum protein (g/dL)	Serum albumin (g/dL)	Proteinuria (mg/24 h)	Serum creatinine (mmol/L)	Serum urea (mmol/L)
Control	9	65 ± 1 (65, 63-66)	39 ± 1 (39, 37-42)	2 ± 1 (2, 1-2)	43 ± 3 (40, 36-48)	7 ± 0 (7, 6-8)
PON d 2 C6 ⁺	6	98 ± 3 ¹ (97, 92-102)	74 ± 1 ¹ (74, 73-76)	857 ± 176 ¹ (972, 679-1080)	97 ± 17 ¹ (86, 67-117)	27 ± 5 ¹ (23, 17-38)
PON d 2 C6 ⁻	5	97 ± 5 ¹ (99, 93-104)	74 ± 4 ¹ (76, 71-79)	789 ± 214 ¹ (788, 11-1145)	117 ± 31 ¹ (97, 64-177)	35 ± 11 ¹ (21, 17-58)
PON d 4 C6 ⁺	6	101 ± 2 ¹ (102, 97-109)	73 ± 1 ¹ (75, 72-76)	1214 ± 131 ¹ (1094, 1013-1197)	93 ± 9 ¹ (94, 80-125)	24 ± 2 ¹ (25, 20-30)
PON d 4 C6 ⁻	6	96 ± 2 ¹ (95, 89-98)	70 ± 1 ¹ (69, 67-73)	1026 ± 267 ¹ (965, 724-1719)	76 ± 5 ¹ (73, 65-85)	19 ± 1 ¹ (18, 17-21)
PON d 8 C6 ⁺	8	97 ± 3 ¹ (97, 88-101)	68 ± 3 ¹ (70, 64-72)	1177 ± 70 ¹ (1165, 709-1321)	74 ± 6 ¹ (65, 53-81)	24 ± 3 ¹ (20, 15-29)
PON d 8 C6 ⁻	9	97 ± 2 ¹ (99, 95-101)	70 ± 3 ¹ (71, 70-73)	1227 ± 116 ¹ (1228, 952-1529)	69 ± 6 ¹ (73, 52-86)	22 ± 3 ¹ (19, 17-28)

Data are expressed as mean ± SEM (median, Inter-quartile range); ¹P < 0.05 compared to control group. PON: Protein-overload nephropathy.

Table 2 Body and kidney weight in the experimental groups

Group	n	Day 1 BW (g)	Final BW (g)	KW (g)	KW:BW
Control	9	186 ± 21 (196, 158-200)	182 ± 5 (192, 168-195)	0.60 ± 0.02 (0.62, 0.53-0.66)	0.33 ± 0.01 (0.33, 0.31-0.34)
PON d 2 C6 ⁺	6	179 ± 2 (168, 164-173)	176 ± 4 (174, 168-184)	1.11 ± 0.05 ¹ (1.06, 1.03-1.22)	0.63 ± 0.03 ¹ (0.62, 0.55-0.72)
PON d 2 C6 ⁻	5	174 ± 3 (167, 159-170)	169 ± 2 (171, 166-176)	1.07 ± 0.11 ¹ (1.20, 0.81-1.26)	0.63 ± 0.07 ¹ (0.67, 0.47-0.75)
PON d 4 C6 ⁺	6	172 ± 2 (181, 174-187)	167 ± 3 (169, 161-173)	1.20 ± 0.07 ¹ (1.29, 1.19-1.35)	0.72 ± 0.05 ¹ (0.76, 0.74-0.84)
PON d 4 C6 ⁻	6	177 ± 9 (165, 176-190)	175 ± 3 (177, 167-183)	1.10 ± 0.05 ¹ (1.09, 0.95-1.11)	0.62 ± 0.02 ¹ (0.60, 0.56-0.67)
PON d 8 C6 ⁺	8	167 ± 4 (165, 159-176)	163 ± 4 (160, 157-173)	1.33 ± 0.08 ¹ (1.30, 1.24-1.38)	0.82 ± 0.05 ¹ (0.80, 0.70-0.88)
PON d 8 C6 ⁻	9	167 ± 3 (165, 160-172)	158 ± 2 (158, 152-166)	1.32 ± 0.22 ¹ (1.30, 1.11-1.57)	0.83 ± 0.04 ¹ (0.78, 0.72-0.95)

Data are expressed as mean ± SEM (median, inter-quartile range); ¹P < 0.05 compared to control group. PON: Protein-overload nephropathy; BW: Body weight; KW: Kidney weight.

dipstick analysis within 24 h of BSA injection, and by quantitation, peaked between days 4 and 8 (Table 1). There were no differences, in either the serum total protein and albumin or proteinuria, between the C6⁺ and C6⁻ groups with PON at any time-point.

Renal dysfunction is similar in C6⁻ and C6⁺ rats with PON

Both the serum urea and creatinine increased in C6 sufficient rats with PON, peaking between days 2 and 4, and beginning to decline by day 8. These changes were not altered by C6 deficiency (Table 1).

Kidney enlargement and TEC proliferation are similar in C6⁻ and C6⁺ rats with PON

Kidney weight increased by more than two-fold in PON, peaking on day 8, and was not altered by C6 deficiency (Table 2). In PON, TEC proliferation increased in both proximal and distal tubules, as assessed by either PCNA or BrdU immunohistochemistry (Table 3, Figures 2 and 3). In the C6 sufficient group, quantitative analysis

showed that the renal cortical expression of PCNA peaked on day 4 and declined on day 8 in PON. The number of BrdU positive cells increased by more than 50-fold, peaking on day 2 (earlier than PCNA) and declining on days 4 and 8. The number of BrdU positive TECs and cortical PCNA expression were both strongly correlated with the serum creatinine (Spearman Rho 0.75 and 0.85 respectively, both *P* < 0.001). There was a trend for a reduction in PCNA and BrdU staining in the C6⁻ group compared to the C6⁺ on day 4, but this did not reach statistical significance (*P* = 0.09 and 0.10 respectively). There were no differences in PCNA or BrdU between C6⁺ and C6⁻ PON groups at any other time-point.

TEC apoptosis is similar in C6⁻ and C6⁺ rats with PON

In PON, the number TUNEL positive TECs increased and this was statistically significant in Field 4 (or the inner cortex/outer medulla) (*P* = 0.03 compared to the control group) whereas there was a trend for an increase in Field 1 (subcapsular cortex) (*P* = 0.15). These data

Table 3 Markers of proliferation and apoptosis in the experimental groups

Group	n	Cortical PCNA (%)	BrdU + TECs (cells/mm ²)	TUNEL + TECs (Field 1)	TUNEL + TECs (Field 4)
Control	9	0 ± 0 (0, 0-0)	0.2 ± 0.0 (0.2, 0-0.2)	6.9 ± 2.5 (8, 0-11.2)	2.7 ± 2.1 (0, 0-5.6)
PON d 2 C6 ⁺	6	0.24 ± 0.06 ¹ (0.28, 0.13-0.36)	8.8 ± 2.7 ¹ (8.9, 2.7-15.3)	-	-
PON d 2 C6 ⁻	5	0.21 ± 0.07 ¹ (0.18, 0.14-0.30)	6.8 ± 3.1 ¹ (6.4, 0.6-13.2)	-	-
PON d 4 C6 ⁺	6	0.34 ± 0.08 ¹ (0.30, 0.09-0.48)	4.6 ± 0.8 ¹ (5.2, 3.4-7.4)	-	-
PON d 4 C6 ⁻	6	0.19 ± 0.04 ¹ (0.18, 0.14-0.30)	3.2 ± 0.5 ¹ (1.2, 1.7-3.7)	-	-
PON d 8 C6 ⁺	8	0.15 ± 0.05 ¹ (0.11, 0.03-0.16)	2.4 ± 0.7 ¹ (3.1, 0-4.1)	13.4 ± 3.3 (12.8, 5.6-16.8)	10.0 ± 2.6 ¹ (9.6, 5.6-17.6)
PON d 8 C6 ⁻	9	0.09 ± 0.03 ¹ (0.08, 0.02-0.11)	2.0 ± 0.7 ¹ (1.2, 0.1-3.7)	11.4 ± 3.7 (9.6, 0-17.6)	7.8 ± 2.8 (6.4, 0-16)

Data are expressed as mean ± SEM (median, Inter-quartile range); ¹P < 0.05 compared to control group; Field 1 was the sub-capsular cortex; Field 4 was the inner cortex/outer medulla. PCNA: Proliferating cell nuclear antigen; TUNEL: Transferase-mediated nick end-labeling; PON: Protein-overload nephropathy; TEC: Tubular epithelial cells.

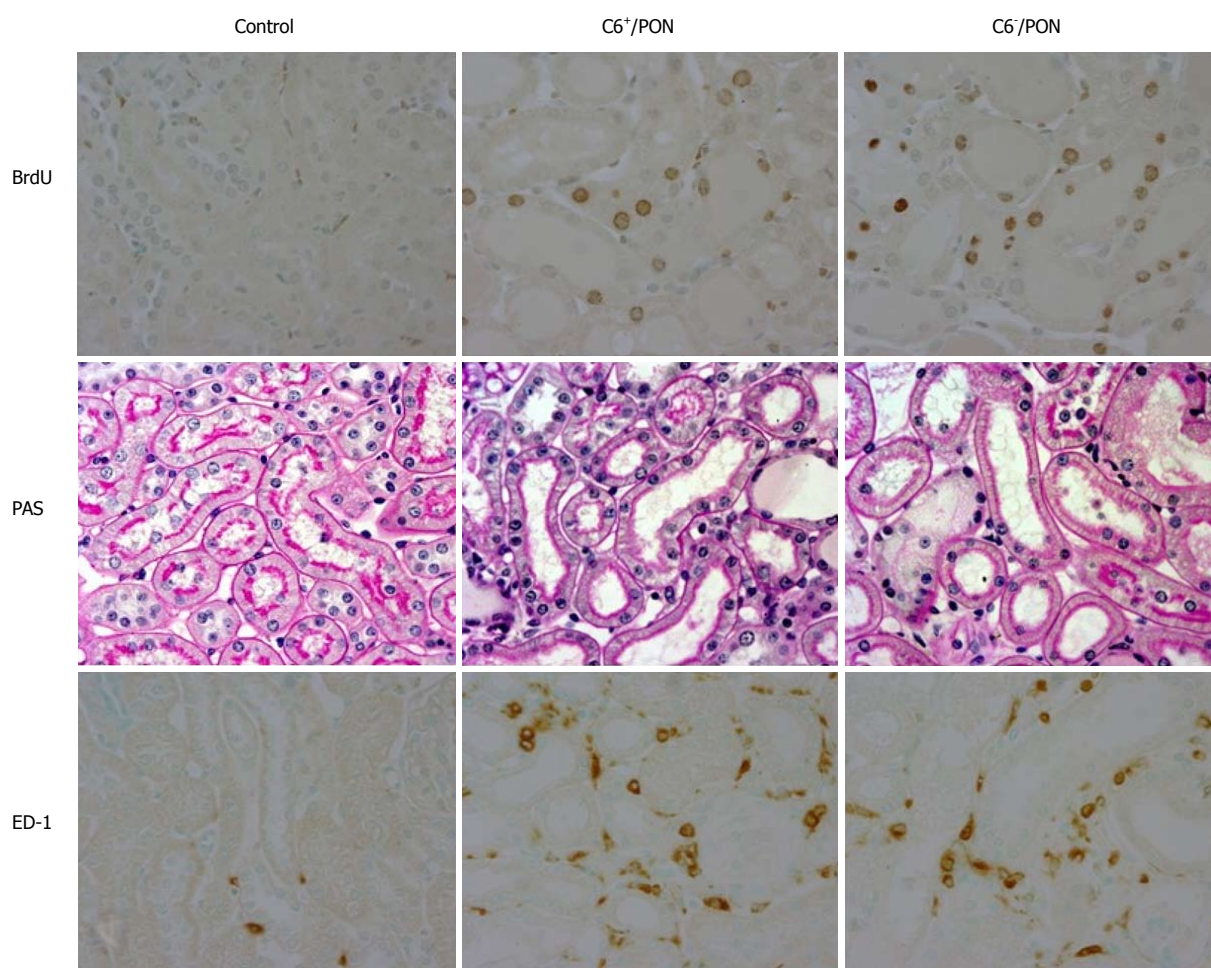


Figure 2 Cortical tubulointerstitial injury in control and protein-overload nephropathy groups on day 8. Representative renal cortical sections (magnification × 400) show immunostaining for BrdU, periodic acid-schiff and ED-1.

are consistent with previous studies on the changes in TEC apoptosis in this model^[38]. However, the increase in TUNEL positivity in TECs in Field 4 was not altered by C6⁻ deficiency (Table 3).

Tubulointerstitial injury is similar in C6⁻ and C6⁺ rats with PON

TEC injury and atrophy, tubular dilatation and distal protein cast formation are the histopathological features

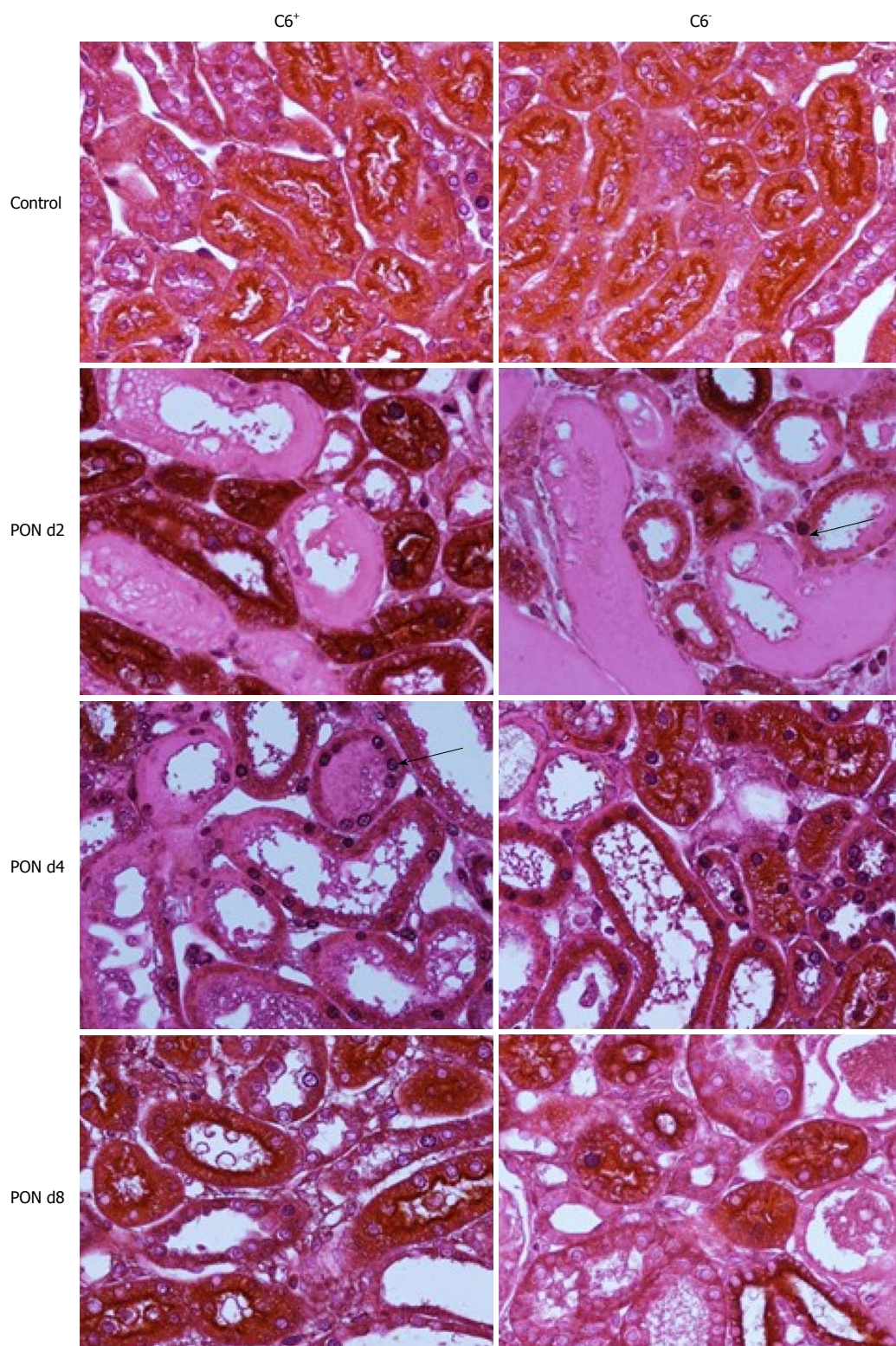


Figure 3 The renal cortical expression of proliferating cell nuclear antigen is increased in protein-overload nephropathy. Representative sections ($\times 200$ magnification) are shown. PCNA positive TEC nuclei are labeled purple (Nickel-enhanced DAB-positive, arrows). Brush border are labeled with anti-Fx1A (brown) and counterstained with periodic acid-schiff. PCNA: Proliferating cell nuclear antigen; TEC: Tubular epithelial cells.

of PON^[41]. Quantitative morphometric analysis was undertaken to precisely determine whether these parameters were altered by C6 deficiency. TEC injury and atrophy was assessed by the cross-sectional height of TECs and vimentin staining. In PON, the mean TEC

height decreased on days 2 and 4, and increased on day 8 (Table 4). On day 8, tubules were noted to have more brush border, accounting for the rise in the TEC height. TEC injury was also assessed by vimentin staining. In control animals, vimentin was present in

Table 4 Assessment of tubulointerstitial injury in the experimental groups

Group	n	TEC height	TEC diameter	Protein cast/field	Interstitial ED-1/field
Control	9	82.3 ± 1.7 (83.3, 78.0-87.0)	253.0 ± 3.7 (256.6, 241.7-259.0)	0	9.4 ± 0.5 (9.3, 8.4-10.6)
PON d 2 C6 ⁺	6	70.9 ± 5.2 ¹ (69.4, 59.5-79.6)	312.1 ± 13.9 ¹ (311.8, 279.4-343.4)	-	20.9 ± 1.6 ¹ (20.1, 17.3-25.5)
PON d 2 C6 ⁻	5	76.6 ± 2.8 ¹ (76.1, 66.6-80.6)	300.8 ± 8.2 ¹ (308.7, 292.7-316.6)	-	14.5 ± 4.0 (11.3, 9.3-17.6)
PON d 4 C6 ⁺	6	68.0 ± 2.3 ¹ (68.3, 60.8-71.5)	309.0 ± 11.1 ¹ (296.8, 291.1-341.1)	2.9 ± 0.5 (3.4, 2.8-3.7)	29.6 ± 2.3 ^{1,2} (28.3, 25.4-38.2)
PON d 4 C6 ⁻	6	67.8 ± 3.8 ¹ (69.3, 61.1-75.4)	303.2 ± 12.6 ¹ (313.0, 275.7-324.2)	2.2 ± 0.6 (1.5, 1.1-3.4)	21.0 ± 1.8 ¹ (20.9, 16.7-23.9)
PON d 8 C6 ⁺	8	74.7 ± 2.7 ^{1,2} (76.2, 71.0-78.9)	304.0 ± 9.2 ¹ (295.5, 280.4-319.1)	1.1 ± 0.3 ³ (0.7, 0.5-1.4)	73.5 ± 6.7 ^{1,2} (78.0, 59.2-85.7)
PON d 8 C6 ⁻	9	70.0 ± 2.9 ¹ (67.7, 63.3-75.3)	298.0 ± 3.8 ¹ (298.6, 289.8-308.1)	1.5 ± 0.5 (1.2, 0.5-2.3)	56.1 ± 7.8 ¹ (56.7, 38.9-80.1)

Data are expressed as mean ± SEM. The units for TEC height and TEC diameter are in arbitrary units. ¹*P* < 0.05 compared to control (median, Inter-quartile range); ²*P* < 0.05 when compared to PON d 2, C6⁺; ³*P* < 0.05 compared to PON d 4, C6⁺. PON: Protein-overload nephropathy; TEC: Tubular epithelial cell.

endothelial cells of glomeruli and peritubular capillaries. In PON, this distribution was preserved, but in addition, there was more prominent peritubular capillary staining and rarely vimentin-positive spindle-shaped cells were noted around tubules on day 8. However, vimentin-positive tubules were not detected during the time-points of the study in PON. In addition, there were no qualitative differences in vimentin staining between the C6⁺ and C6⁻ groups with PON (data not shown).

The mean cross-section tubule diameter increased in a time-dependent manner, peaking on days 2 to 4 and decreasing on day 8 (Table 4). There was a strong relationship between tubular diameter and serum creatinine (Spearman Rho 0.85, *P* < 0.001), as well as the number of BrdU positive TECs and renal cortical PCNA expression (Spearman Rho 0.83 and 0.80 respectively, both *P* < 0.001). In addition, the number of proteinaceous casts in distal tubules increased in PON, peaking on day 4 and declining slightly by day 8. However, neither tubule diameter or cast formation was affected by complement deficiency in PON (Table 4).

Interstitial monocyte accumulation increased progressively in PON in a time-dependent manner peaking on day 8, when it more than 7-fold higher than the control group. There was a partial reduction in interstitial ED-1 accumulation in C6⁻ animals with PON which reached statistical significance only on day 4 (*P* = 0.01 compared to C6⁺ PON) but not on days 2 (*P* = 0.10 compared to C6⁺ PON) and 8 (*P* = 0.15, compared to C6⁺ PON).

In control animals, α-SMA was constitutively present in capillaries and this distribution was not altered in PON. Rarely, on day 8, some α-SMA positive cells were present around dilated collecting tubules, representing early foci of fibrosis. However, there were no qualitative differences in complement sufficient animals (data not shown).

DISCUSSION

The results of this study provide further important

refinements about the role of the complement membrane attack complex (C5b-9) in non-immunological mediated chronic kidney disease models characterized by nephrotic-range proteinuria. First, in contrast to chronic proteinuric models (PAN, RK and AN)^[16,17,32], C5b-9 deposition in acute and short-term PON was localized predominantly to the basolateral membrane of TECs and virtually undetectable on the lumen of TECs. The reasons for this difference have not been investigated in the present study but it presumably relates to the pattern of glomerular injury and selectivity of the proteinuria in PON, given that properdin remains detectable on the apical membrane of TECs in this model^[28]. Similar to minimal change disease in humans (in which urinary and renal C5b-9 is not increased)^[6,33,35], the early stage of PON is characterized by highly selective proteinuria, predominantly albuminuria (consisting of both heterologous and autologous albumin)^[36,42] with marginal changes in glomerular size permselectivity^[43]. For example, in PAN, albumin constitutes 57% of the protein excreted in the urine whereas in PON, this is significantly higher at 90%^[36]. In addition, it is also possible that large amounts of albumin in the tubular lumen may also, in some way, minimize the density of complement proteins needed to assemble C5b-9^[6].

Moreover, in divergence to previous studies using other models of chronic proteinuria (PAN, RK, AN)^[16,17,32], tubulointerstitial injury was not altered by C6 deficiency in PON. In the present study, tubulointerstitial injury was assessed by several methods, including TEC proliferation and apoptosis, morphometric assessment and interstitial ED-1 accumulation. There was a trend for a reduction in TEC proliferation (as assessed by BrdU and PCNA) on day 4 and no significant changes were seen at any other time-point. In addition there were no differences detected in TEC injury, as objectively assessed by morphometric assessment. Although a reduction in interstitial monocyte accumulation was detected on day 4, this was insufficient to alter either TEC injury or renal dysfunction. The predominant basolateral deposition of

C5b-9 could explain the neutral effects of C6 deficiency on tubulointerstitial injury in PON in the present study. This is supported by previous studies in which C6 deficiency does not alter the progression of non-proteinuric models of chronic kidney disease^[31], and the observation that CD59 (a membrane bound inhibitor of C5b-9) is located on the basolateral region of TECs and absent on the lumen^[44-46].

The PVG C6 deficient rat strain was a chance discovery made by Leenaerts *et al*^[40] who first described the specific defect in the complement system in these animals. The key abnormality is a partial and isolated defect of C6 (unstable mRNA or point mutation) due to a spontaneous autosomal recessive genetic defect^[47]. The abnormality was restricted to PVG rats obtained from Bantin and Kingman (Fremont, CA, United States) and not present in rats from other animal vendors (Harlan SD, Harlan Olac, others)^[40]. In the C6 deficient strain the activation of the complement system proceeds normally to the level of C5, and thus the generation of opsonic C3b and chemotactic C5a are not known to be affected^[40]. The C6 deficient strain are not susceptible to immunocompromised infections^[40], have normal T-cell responses^[48] and their tissue antigenic expression and immunity are identical to the C6 sufficient PVG strain^[40,48]. The exact mechanisms underlying the tubulointerstitial localisation of C5b-9 to the basolateral region in C6⁺ rats with PON in the present study, are not clear but previous evidence would suggest that it is a consequence of non-immunological mediated TEC injury, intra-renal complement synthesis and presumably a sequela of interstitial oedema and inflammation in this model^[49-51]. The TEC injury and interstitial inflammatory response in PON is postulated to be secondary to "proteinuria-induced TEC injury" rather than immunologic factors^[14]. The PON model does not show evidence of tubulointerstitial rat IgG or circulating antibodies to BSA^[14].

Abbate and colleagues reported that C3 mediates both glomerular as well as tubulointerstitial injury in mice with PON^[52]. In the latter study, C3 deficiency attenuated both proteinuria and tubulointerstitial injury in PON^[52]. Furthermore, the latter study also demonstrated that PON-mediated renal injury was only partially attenuated in C3^{-/-} kidneys transplanted into wild-type recipients, suggesting that circulating C3 may be more critical to the pathogenesis of PON than the local generation of C3 within kidney^[52] (though this finding may be specific to PON as it was not the situation in AN^[53]). In any case, the results of the current study suggest that C6 is not likely to be a critical down-stream mediator of C3-induced renal injury in PON, and emphasise potential hierarchical roles for other complement factors, such as C3b and/or C5a^[46,52,53] in this setting.

In contrast to the findings of the current study, Eddy and colleagues previously detected C5b-9 in the tubular lumen in PON^[14]. The different results obtained in our study could be due to the lower dose of BSA and proteinuria (two-fold less than the current study) as well

as the use of uninephrectomy in the model described by Eddy *et al*^[14]. These factors may alter the pattern of the glomerular injury and selectivity of proteinuria^[54], and influence the formation of C5b-9 in the tubular lumen^[17].

In this study, the time-dependant changes of tubulointerstitial injury in PON were quantitated precisely, for the first time, by morphometric analysis. These data highlight some important pathophysiological insights into the nephron response to excess luminal proteins. Initially, we found that the TEC height decreased on day 2 but increased on day 8. The increase in TEC height on day 8 was temporally associated with a focal increase in the number of proximal TECs expressing brush border and a reduction in the mean serum creatinine. These changes were preceded by a dramatic increase in kidney weight and TEC proliferation, which is a consistent feature of nephrotic glomerular disease in human biopsy studies^[55,56]. Taken together, these data suggest that the transient increase in TEC proliferation in acute nephrotic glomerular disease may be a compensatory mechanism that leads to nephron hypertrophy and attempts to restore renal function as well as provide an increased surface area to absorb the excess luminal proteins. This hypothesis is also supported by data showing that inhibition of TEC proliferation by rapamycin causes cast nephropathy and acute renal failure in PON^[41]. The current study also highlights non-complement dependant mechanisms of tubulointerstitial injury in glomerular diseases, particularly the adverse effects of intratubular obstruction due to protein-cast nephropathy^[37], as detected by the increase in cross-section tubule diameter in PON.

Bearing in mind the limitations of PON as an animal model, the results of the current preclinical study have the strongest implications for pathogenesis of early renal injury associated with human minimal change disease. So far, only a few studies in humans have compared the pattern of complement expression in minimal change disease with other types of glomerulonephritides^[6]. Based on clinical experience from renal biopsy samples minimal change disease is not believed to be mediated by C5b-9^[34]. Furthermore, despite the detection of properdin on the apical brush border of proximal tubules in rats with PON^[28], the urinary excretion of C5b-9 in human minimal change disease is low^[6,33]. Taken together, these findings could provide a potential explanation for the lower incidence of end-stage kidney disease in minimal change nephropathy in comparison to other types of nephrotic glomerular diseases^[57].

The results of this study have a number of important limitations. First, the animal model of PON does not exactly replicate human glomerular disease, in that injections of heterologous albumin are required to induce selective proteinuria, and this has systemic effects^[58] which are not present in the human counterpart. Second, further studies are required to confirm and define the mechanisms underlying the absence of luminal C5b-9 formation in acute PON. In this regard, the urinary excretion of C5b-9 as well as other

complement regulatory proteins (particularly properdin and Factor H)^[29,30,59] in PON could be compared to other chronic proteinuric and non-proteinuric models of chronic kidney disease as well as different types of proteinuric diseases in humans, in future studies. Finally, the present study has only examined the acute stages of PON, and different mechanisms of C5b-9-mediated injury could be involved if it is combined with renal mass reduction and/or if the injections of BSA are continued for a longer duration^[14].

In conclusion, the results of the present study shows that C5b-9 does not mediate tubulointerstitial injury in acute short-term PON, and this may be due to the low level of luminal C5b-9 formation. Taken together with the results of previous studies using C6 deficient animals in chronic proteinuric renal disease models^[16,17,32], these data suggest that the selectivity of proteinuria may be an important factor in the causation of tubulointerstitial damage in nephrotic glomerular diseases. Specifically, in the case of acute PON, the pathogenesis of tubulointerstitial injury is C5b-9-independent and the tubular filtration of excess albumin, growth factors and microtubular protein-cast obstruction are likely to be more critical^[37]. Further studies to understand the role of complement system will be helpful in defining new therapies for the generic treatment of kidney diseases characterized by chronic proteinuria^[29].

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COMMENTS

Background

In humans with chronic glomerular disease, the degree of tubular atrophy and interstitial disease are the strongest histological parameters which correlate with renal function and predict progression.

Research frontiers

The proteinuria in protein-overload nephropathy (PON) is almost immediate in onset and highly selective in composition.

Innovations and breakthroughs

Interstitial monocyte (ED-1⁺ cell) accumulation was partially reduced in C6⁻ animals with PON on day 4 ($P = 0.01$) but there was no change in myofibroblast accumulation.

Applications

The results of this study provide further important refinements about the role of the complement membrane attack complex (C5b-9) in non-immunological

mediated chronic kidney disease models characterized by nephrotic-range proteinuria.

Terminology

PON was induced in adult female Piebald-Viral-Glaxo rats with or without complement C6 deficiency (C6⁻ and C6⁺) by daily intraperitoneal injections of bovine serum albumin (2 g/d), and examined on days 2, 4 and 8.

Peer-review

This paper is well written and has interesting findings.

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