Kidney International, Vol. 62 (2002), pp. 2010–2021

Decay-accelerating factor expression in the rat kidney is restricted to the apical surface of podocytes

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Background. Decay-accelerating factor (DAF) has inhibitory activity toward complement C3 and C5 convertases. DAF is present in human glomeruli and on cultured human glomerular visceral epithelial cells (GEC). We studied the distribution and function of rat DAF.

Methods. Function-neutralizing antibodies (Abs) were raised against DAF. The distribution of DAF in vivo was determined by immunoelectron microscopy. Functional studies were performed in cultured GEC and following IV injection of anti-DAF Abs into rats.

Results. DAF was present exclusively on the apical surfaces of GEC, and was not present on the basal surfaces of GEC, nor other glomerular or kidney cells. DAF was functionally active on cultured GEC, and served to limit complement activation in concert with CD59, an inhibitor of C5b-9 formation. Upon injection into normal rats, anti-DAF $F(ab')_2$ Abs bound to GEC in vivo, yet there was no evidence for complement activation and animals did not develop abnormal albuminuria. Anti-megalin complement-activating IgG Abs were "planted" on GEC, which activated complement as evidenced by the presence of C3d on GEC. Attempts to inhibit DAF function with anti-DAF Abs did not affect the quantity of complement activation by these anti-megalin Abs, nor did it lead to development of abnormal albuminuria. In contrast, in the puromycin aminonucleoside model of GEC injury and proteinuria, anti-DAF Abs slowed the recovery from renal failure that occurs in this model.

Conclusion. In cultured rat GEC, DAF is an effective complement regulator. In vivo, DAF is present on GEC apical surfaces. Yet, it appears that DAF is not essential to prevent complement activation from occurring under normal circumstances and in those cases in which complement-activating Abs are present on the basal surfaces of GEC in vivo. However, in proteinuric conditions, DAF appears to be protective to GEC.

Key words: glomerular epithelial cells, complement regulator, Heymann nephritis, puromycin aminonucleoside nephrosis, proteinuria, renal injury.

Received for publication October 1, 2001
and in revised form June 10, 2002
Accepted for publication July 8, 2002

Complement activation can proceed via either the alternative, classical or mannose-binding lectin pathways [1, 2]. Activation through each of these pathways leads to cleavage of C3 with generation of the proinflammatory and regulatory fragments, C3a and C3b. C3b attaches covalently to immune complexes, which is followed by C5 binding and its cleavage to C5a and C5b. The former is a potent inflammatory molecule that can recruit and activate neutrophils and monocytes, while the generation of C5b begins the non-enzymatic assembly of the C5b-9 membrane attack complex that can result in cellular death or activation following membrane insertion [3].

To prevent injury of self-tissue, complement is regulated by both plasma and cell membrane-associated proteins [4]. A focal point of regulation is at the level of the C3/C5 convertases of both pathways. This occurs in humans via the action of the plasma proteins, factor H and C4-binding protein, and the cell membrane proteins, complement receptor 1 (CR1), decay-accelerating factor (DAF), and membrane cofactor protein, all members of the regulators of complement activation gene family [5]. These proteins inhibit C3/C5 convertases by accelerating their intrinsic decay and/or by acting as a factor I cofactor for the cleavage and inactivation of C3b and C4b. The related rodent protein, Crry (CR1-related gene/protein y), has combined decay-accelerating and factor I cofactor activity for C3b and C4b [6, 7].

The complement system is considered to be relevant to a number of diseases affecting the glomerulus [8]. In most cases of immunologically-mediated glomerular diseases, complement components are found in diseased glomeruli. Furthermore, in some disease states, such as

lupus nephritis, postinfectious glomerulonephritis (GN), and membranoproliferative GN, systemic complement consumption is evident. A considerable amount of experimental evidence also has been accumulated from animal models that has shed additional light on the role for complement activation in human glomerular diseases [9].

One of the most thoroughly studied glomerular disease models and for which data have been accumulated implicating a role for the complement system is the Heymann nephritis (HN) model of membranous nephropathy [10]. In the passive HN model, injected anti-Fx1A antibodies (Abs) bind to megalin and other visceral glomerular epithelial cell (GEC) antigens [11–13]. When a threshold amount of Abs has accumulated, complement activation can proceed, C5b-9 is generated, and GEC injury occurs [14–17]. As the GEC appears to be key to the integrity of the glomerular permselectivity barrier, an acquired or inherited abnormality of the GEC is sufficient to lead to the development of abnormal proteinuria [18], as occurs in passive HN. Consistent with this theme is that the relatively specific toxin for GEC, puromycin aminonucleoside (PAN) leads to marked proteinuria in the PAN nephrosis model [19, 20].

There is a balance between complement activation and its regulation [21]. By histological techniques, human glomeruli contain all of the regulators of complement activation members, and these may be up-regulated under disease states in which complement is activated [22–24]. In vitro, cultured human GEC bear functionally active DAF [25].

The situation in rat glomeruli appears comparable to that in humans. Crry is present in glomerular cells in vitro and in vivo [26, 27]. In the case of GEC, Crry limits complement activation through both classical and alternative pathways in vitro [6, 7], and is responsible for limiting complement activation in the active HN model in vivo [28]. Originally, investigators in the field considered Crry to replace both DAF and membrane cofactor protein in glomeruli. However, with the identification of rat DAF, it is clear that this assumption was incorrect [29, 30]. In this study, we characterized rat DAF that is exclusively present on GEC in the kidney.

METHODS

Antibodies

Recombinant rat DAF was produced as described previously [30]. A single sheep was immunized with DAF in complete Freund's adjuvant for the primary immunization and incomplete Freund's adjuvant for booster immunizations (Charles River Pharmservices, Southbridge, MA, USA). IgG was purified from pre-immune and immune sera by protein G affinity chromatography (Amersham Pharmacia Biotech, Piscataway, NJ, USA). In some studies, affinity purified anti-DAF produced by affinity chromatography on a column of recombinant DAF bound to CNBr-Sepharose [31] was utilized. Monoclonal mouse anti-rat DAF Ab clone RDIII-7 was generated as previously described [29].

Mouse hybridoma clones K57/27 and K35/9 producing anti-rat megalin (gp330) monoclonal Abs were kindly provided by Dr. Donna Mendrick (Gene Logic, Inc., Gaithersburg, MD, USA) [32]. These clones were cultured and IgG isolated from supernatants by protein G affinity chromatography. The IgG was concentrated and dialyzed into 150 mmol/L NaCl. To confirm the reactivity of the mAbs against megalin, indirect immunofluorescence (IF) staining was performed on normal rat kidney documenting strongly positive proximal tubular brush border staining [32].

Polyclonal sheep Abs to recombinant rat CD59 were raised as previously detailed [31]. Monoclonal anti-rat CD59 clone 6D1 also has been described [33]. Both Abs neutralize the function of CD59 in vitro and in vivo [27, 31]. Rabbit anti-rat erythrocyte Abs were raised and absorbed against recombinant rat CD59 [34]. Rabbit anti-rat GEC Abs were produced as described previously [13].

$F(ab')_2$ fragments of sheep anti-DAF, anti-CD59 and pre-immune IgG were obtained by pepsin digestion followed by size exclusion chromatography [28]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the purity of all IgG and $F(ab')_2$ Abs.

Functional studies

Rat erythrocytes were sensitized by incubation (15 min, 37°C) with a 1/100 dilution in phosphate-buffered saline (PBS) of anti-erythrocyte Abs. Blocking anti-rat CD59 mAb 6D1 (10 μg/mL) and/or affinity-purified sheep anti-rat DAF $F(ab')_2$ (10 μg/mL) were included in the sensitization step. Sensitized erythrocytes were washed into 0.14 mol/L NaCl, 0.01 mol/L Na barbital, pH 7.4 (VBS) and incubated (30 min, 37°C) with serial dilutions of normal rat serum (NRS) diluted in VBS. Hemoglobin released into the supernatants was expressed as a percentage of that maximally released by H_2O.

Rat GEC were cultured and studied for complement regulation as previously described [27]. In this technique, the release of the previously cell-incorporated fluorescent probe, biscarboxyethyl carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR, USA) was used as a marker of complement-mediated cytotoxicity. Cells were loaded with the acetoxymethyl ester of BCECF and subsequently exposed to rabbit anti-GEC IgG at 1 mg/mL in VBS. In this step, some cells were also exposed to sheep anti-DAF and/or anti-CD59 $F(ab')_2$ at 0.3 mg/mL in VBS. Subsequently, cells were incubated in 40% NRS for 40 minutes at 37°C, and BCECF released into the supernatants was measured spectrofluorimetrically and expressed as a percentage of that maximally released by

melittin (50 μg/mL; Sigma Aldrich, St. Louis, MO, USA). Control cells were treated identically, yet not exposed to Ab nor to serum, and were used to determine the spontaneous leakage of BCECF [35].

Animal experiments

All work with animals was approved by the University of Chicago Animal Care and Use Committee and were performed in accord with the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing 225 to 250 grams were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Inhalational isoflurane was used to anesthetize rats during all procedures.

In the first group of studies, anti-rat DAF $F(ab')_2$ Abs were injected intravenously into normal rats. To examine the localization of injected anti-DAF, whether intrinsic DAF might be modulated, and if neutralization of DAF function affected the glomerular permselectivity barrier to protein passage, groups of two rats each were injected IV with 10, 20 and 40 mg anti-rat DAF $F(ab')_2$. Animals were studied within 24 hours of Ab injection, with the rationale that injected $F(ab')_2$ Abs would have ready access to the GEC in vivo [36]. Within four hours of Ab injection, animals were housed for 24 hours in metabolic cages for urine collection, and then sacrificed for blood and tissue harvest.

In the second group of studies, the role of DAF in passive HN was determined. For these studies, 15 mg each of anti-megalin monoclonal Abs K57/27 and K35/9 were injected into rats. As is known from past studies by Salant et al [17] and verified in our prior studies with these anti-megalin Abs [31], there is progressive glomerular accumulation of anti-megalin Abs over time. By five days following injection, significant subepithelial immune deposits and immunoreactive C3d are present, the latter evidence for complement activation, yet, there is not increased proteinuria, unlike passive HN induced by anti-Fx1A Abs [31]. The role for DAF in limiting complement activation in the setting in which anti-megalin Abs were present on GEC was studied by injecting four animals with 20 mg anti-DAF $F(ab')_2$. Control animals ($N = 4$) were treated identically except they received pre-immune $F(ab')_2$ instead of anti-DAF. Because in vitro studies in erythrocytes and GEC suggested that even with DAF neutralization, complement activation was limited by functional CD59 (see below), a third group of animals with GEC-bound anti-megalin Abs received 20 mg anti-DAF and anti-CD59 $F(ab')_2$. In each of these three groups, following injection of $F(ab')_2$ Abs, urine was collected for 24 hours and animals were then sacrificed.

In a third group of studies, the role of DAF was studied in the PAN nephrosis model that was induced by a single IP injection of 15 mg PAN (Sigma) per 100 g body weight [37]. On the eighth day of disease, all animals underwent survival renal biopsies to determine intrinsic DAF distribution, and then animals were given either 20 mg sheep anti-DAF ($N = 4$) or pre-immune sheep IgG ($N = 5$). Survival renal biopsies were performed 9 and 10 days after PAN injection ($N = 2$ to 3 in each group) to determine intrinsic DAF and sheep anti-DAF Ab distribution. Serum and urine samples were collected at selected times from days 0 to 11. On the eleventh day after PAN injection, all rats were sacrificed and renal tissue was harvested.

Measurements from blood and urine

Blood was first drawn into a heparinized micro-hematocrit capillary tube for hematocrit determination, followed by collection for serum isolation. Blood urea nitrogen (BUN) and urinary creatinine concentrations were measured with a Beckman Autoanalyzer (Beckman Instruments, Fullerton, CA, USA). Urinary albumin was measured by a previously described enzyme-linked immunosorbent assay (ELISA) technique [38]. Urinary excretion of albumin was normalized to urinary creatinine and expresses as μg albumin/mg creatinine. Urinary protein was measured with sulfosalicyclic acid [28] and data are presented as mg protein per 24 hours.

Tissue processing

Portions of renal tissue were formalin fixed and embedded in HistoPrep (Fisher Scientific, Pittsburgh, PA, USA) and snap frozen in isopentane on dry ice. For light microscopy, 4 μm sections were stained with periodic acid Schiff and provided as coded slides to a renal pathologist (MH). In the PAN studies, the percentage of total glomerular area occupied by sclerosis and/or hyalinosis was estimated. In addition, the grading of tubular changes was done using a modification of the protocol of Nomura et al [39]. The extent of cast formation, dilation and degenerative changes in tubules in the cortex and outer stripe of the outer medulla was quantified on a 0 to 3 scale, in which 0 was no involvement; 0.5 = $<$10%; 1 = 10 to 20%; 2 = 30 to 70%; and; 3 = $>$70% of tubules involved. A composite tubular injury score was arrived at by summing the three individual scores from each animal.

For immunofluorescence (IF) microscopy, 4 μm cryostat sections were directly stained for sheep $F(ab')_2$ and rat C3 using fluorescein isothiocyanate (FITC)-conjugated Abs (Cappel Laboratories, Durham, NC, USA). Since the latter detects only C3c, staining for C3d was done using a specific anti-human C3d Ab (Dako, Carpinteria, CA, USA), which is cross-reactive with rat C3d [40]. An indirect IF procedure was used to detect rat DAF in which sheep anti-rat DAF was followed by FITC-conjugated anti-sheep IgG. For dual detection of rat DAF and sheep anti-DAF $F(ab')_2$ in tissue sections,

FITC-conjugated anti-sheep $F(ab')_2$, monoclonal anti-rat DAF, and rhodamine-conjugated sheep anti-mouse IgG (Cappel) were incubated in succession. A semiquantitative scoring system was used in which each slide was scored from 0 (negative) to 4+ (strongest) using a schema previously published [38]. In all cases, the observer was blinded as to the origin of the slides.

Immunoelectron microscopy

A kidney from an adult Sprague-Dawley rat was collected and fixed with 1% paraformaldehyde-0.05% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.3 for 1½ hours on ice. Fixative was washed out using 0.1 mol/L phosphate buffer, pH 7.3 and tissue was equilibrated in 30% sucrose in buffer. Samples were frozen in OCT using isopentane chilled in a bath of acetone and dry ice. Frozen sections, 30 μm thick, were picked up on Nunc Thermanox coverslips (Nalge Nunc International, Rochester, NY, USA) that were air-dried for two hours and used the same day or stored at 4°C. Sections were blocked with 0.5 mol/L ammonium chloride in PBS for 30 minutes and then with 5% normal rabbit serum in PBS for 30 minutes. Sections were incubated with 15 μg/mL affinity purified sheep anti-DAF $F(ab')_2$ or irrelevant sheep IgG as a control, for one hour at room temperature in a humid chamber, washed with PBS, and incubated with rabbit anti-sheep $F(ab')_2$-HRP (ICN Biomedicals, Inc., Irvine, CA, USA) diluted l:500 for one hour. After washing with PBS, sections were re-fixed with Karnovsky's fixative (using 0.1 mol/L phosphate buffer, pH 7.3) for 15 minutes and then washed using 0.1 mol/L phosphate buffer, pH 7.3. Peroxidase histochemistry was carried out using 0.05% DAB–0.01% H_2O_2 in 0.1 mol/L phosphate buffer, pH 6.0 for one hour. Coverslips were washed with buffer and post-fixed with 2% OsO_4 in 0.1 mol/L phosphate buffer, pH 7.3 for 1½ hours, dehydrated through 100% ethanol, and infiltrated with graded mixtures of ethanol/Polybed 812 into 100% Polybed 812 (Polysciences, Warrington, PA, USA). Samples were polymerized in 35 mm dishes with fresh Polybed 812 overnight at 60°C, peeled from coverslips, and adhered to the flat end of polymerized stubs. Ultrathin sections were stained with Reynold's lead citrate for two minutes and viewed with a JEOL 100CX electron microscope (JEOL, Tokyo, Japan).

Statistics

Statistical analyses were performed with Minitab Software (College Park, MD, USA). Data are expressed as mean ± SEM unless noted otherwise and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Fisher's pair-wise comparisons. P values <0.05 were considered statistically significant.

RESULTS

Localization of DAF in normal rat kidneys

Using an indirect IF technique, DAF was very strongly expressed in rat glomeruli (Fig. 1). Although the staining pattern was consistent with GEC localization of DAF (arrows), the higher resolution technique of immunoelectron microscopy was used to definitively localize rat DAF in kidney. Indirect immunoperoxidase techniques localized DAF specifically to the apical surfaces of podocytes and their foot processes. As shown in Figure 2, electron dense peroxidase reaction product was found exclusively and diffusely on apical membrane domains of podocytes. In contrast, no reaction product was detected on endothelial cell surfaces, within the GBM, or on basal cell surfaces of podocyte foot processes adherent to the GBM (Fig. 2). Similarly, glomerular mesangial cells, tubular epithelial cells, and all tubulointerstitial cells in the kidney were completely negative, as were control tissues incubated with irrelevant sheep IgG (not shown).

Functional role of DAF on rat erythrocytes and cultured GEC

The role of DAF to limit complement activation on rat erythrocytes was studied. In these experiments, classical pathway complement activation was induced by sensitizing erythrocytes with a polyclonal Ab followed by exposure to NRS as a source of complement. As shown in Figure 3, rat erythrocytes were protected from activation of homologous complement. Inhibition of DAF function alone with neutralizing Abs led to a small, but significant increase in hemolysis. Inhibition of CD59 caused a larger increase in hemolysis, but when both DAF and CD59 were neutralized, there was marked increase in hemolysis, consistent with having impaired complement regulation both at the level of C3/C5 convertases and at C5b-9 formation.

Given the prominent expression of DAF in rat GEC in vivo, the complement regulatory activities of DAF in cultured rat GEC were explored. In these studies, both spontaneous alternative pathway activation and Ab-mediated classical pathway activation were studied. Given the absence of megalin on cultured rat GEC, a polyclonal complement-activating Ab was used instead [27]. As shown in Figure 4, GEC effectively regulated complement activation through both pathways. In particular, GEC that were not Ab-sensitized but exposed to serum as a complement source did not release BCECF above that spontaneously released by cells exposed to buffer alone, indicating GEC can completely prevent spontaneous activation of complement through the alternative pathway. When the function of DAF alone was neutralized there was a small enhancement in complement activation. However, when the function of both DAF and CD59 were neutralized on the surface of GEC, there

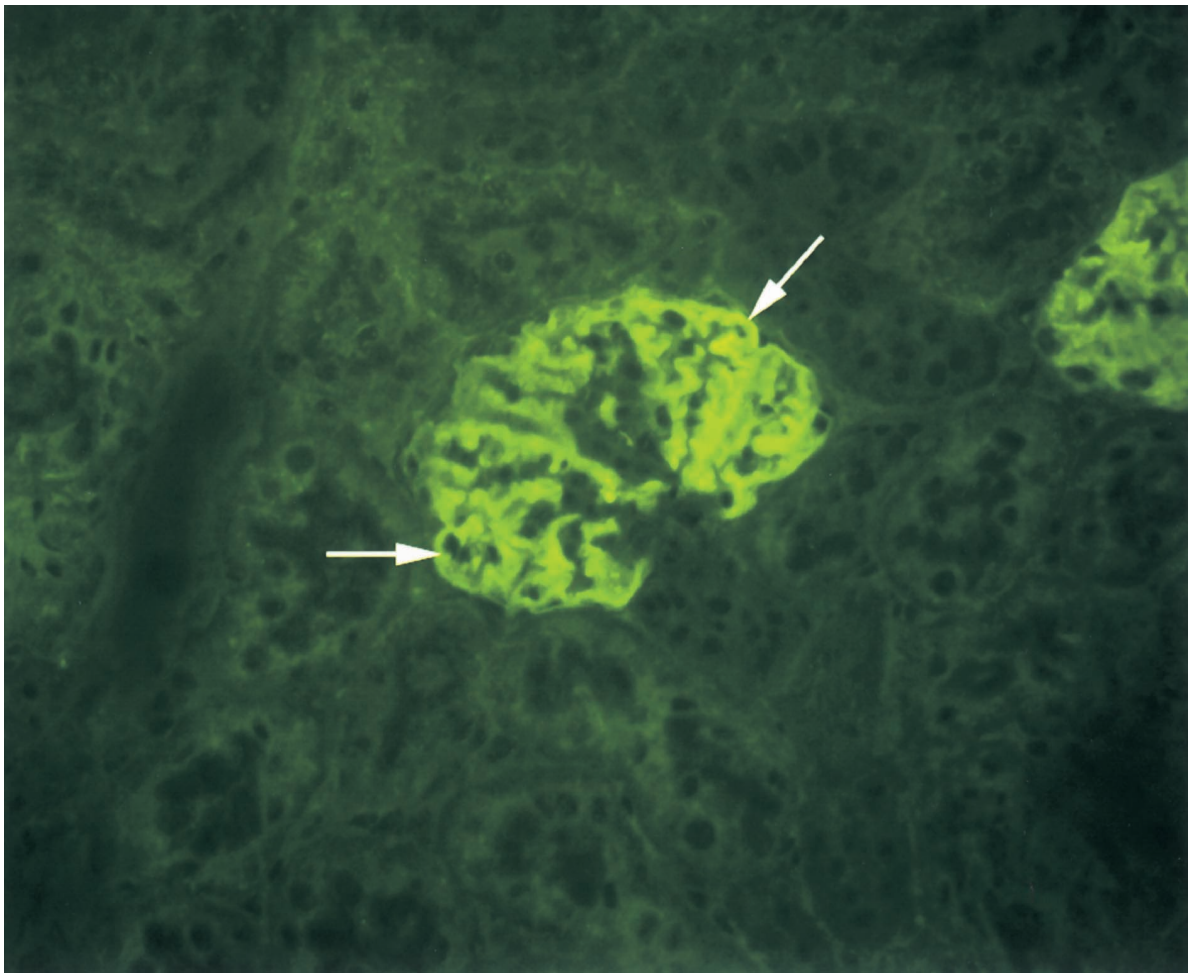

Fig. 1. Distribution of decay accelerating factor (DAF) in rat kidney by immunofluorescence (IF) microscopy. Normal rat kidney was stained with affinity purified sheep anti-rat DAF $F(ab')_2$ followed by FITC-conjugated anti-sheep IgG. The arrows depict apparent glomerular epithelial cell (GEC) staining.

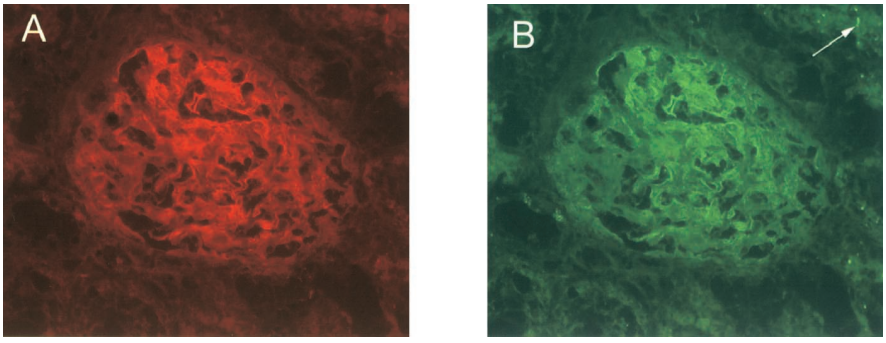

Fig. 5. Immunofluorescence staining for rat DAF (*A*) and sheep $F(ab')_2$ (*B*) 24 hours following injection of 40 mg sheep anti-rat DAF $F(ab')_2$. A dual labeling technique was utilized in which glomerular bound sheep anti-DAF $F(ab')_2$ was detected with a specific FITC-conjugated Ab and intrinsic DAF was identified by a monoclonal anti-DAF followed by a specific rhodamine-conjugated Ab. The arrow depicts tubular resorption droplets of sheep $F(ab')_2$.

was markedly increased cytotoxicity occurring from both classical and alternative pathway complement activation.

Functional role of DAF on GEC in vivo

Initial studies sought to determine the fate of intravenously injected anti-DAF $F(ab')_2$ Abs as well as intrinsic DAF to which the Abs were directed. Preliminary studies indicated that, while injected anti-DAF $F(ab')_2$ could be identified in glomeruli as early as 10 minutes after IV injection, significant Ab binding was present at 24 hours post-injection, consistent with our past studies and those by Salant et al, in which injected $F(ab')_2$ Abs appear to readily cross the glomerular capillary wall accessing the GEC [28, 36]; therefore, this time point was

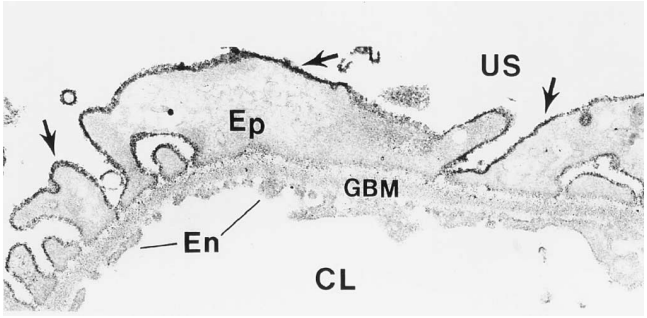

Fig. 2. Ultrastructural distribution of DAF by immunoelectron microscopy. Electron-dense peroxidase reaction product is seen exclusively on the apical membrane surfaces (arrows) of GEC foot processes (Ep). No labeling is detected on endothelial cells (En), within the glomerular basement membrane (GBM), or on basal surfaces of podocytes. Abbreviations are: CL, capillary lumen; US, urinary space.

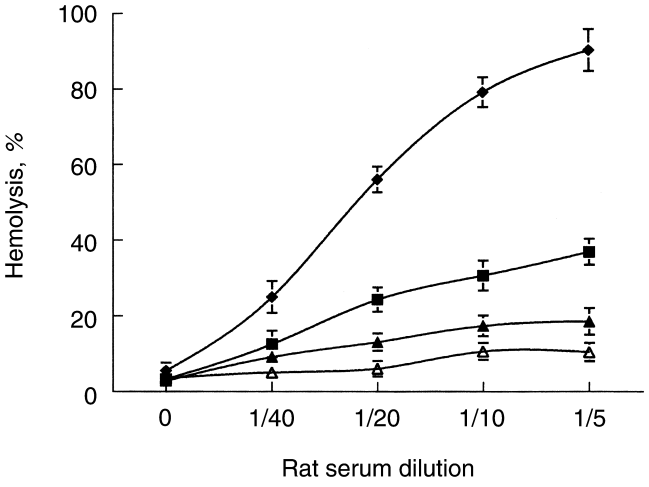

Fig. 3. Effects of neutralizing erythrocyte DAF and/or CD59 on classical pathway-mediated hemolysis. Antibody (Ab)-sensitized erythrocytes were exposed to function-neutralizing anti-DAF and/or anti-CD59 Abs, followed by varying dilutions of NRS as a complement source. The percent of maximal hemolysis was measured. Data are mean ± SD ($N = 3$ at each point). There was a statistically significant difference between each condition at serum dilutions 1/20 and higher. Symbols are: (◆) anti DAF & anti CD59; (■) anti-CD59; (▲) anti-DAF; (△) none.

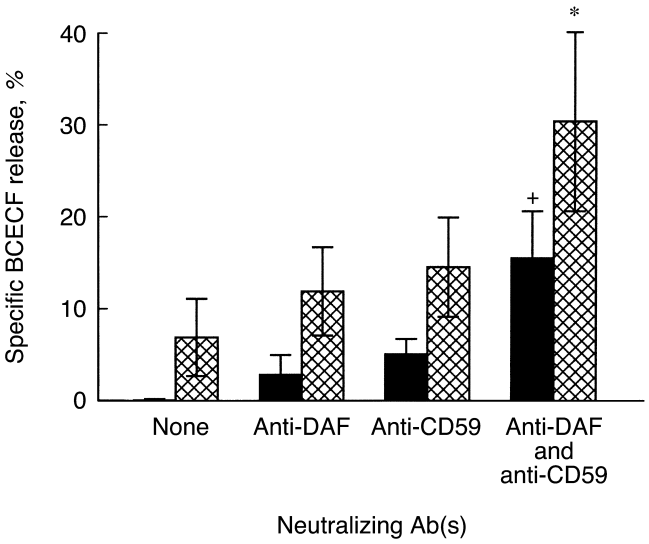

Fig. 4. Effects of neutralization of DAF and/or CD59 on GEC resistance to complement activation. GEC were either not sensitized with Ab (■) to evaluate alternative pathway activation or sensitized with anti-GEC (⊠) to evaluate classical pathway activation. Cells were simultaneously exposed to function-neutralizing anti-DAF and/or anti-CD59 $F(ab')_2$ Abs, followed by 40% NRS as a complement source. The percent of maximal release of BCECF was measured. Shown are data from one experiment, representative of four performed. Each point is mean ± SEM ($N = 3$ at each point). $+P < 0.05$ vs. no Ab; $*P < 0.05$ vs. all other groups.

chosen for subsequent experiments. Groups of two rats each were given 10, 20 or 40 mg anti-DAF $F(ab')_2$, following which urine was collected for 24 hours and animals were then sacrificed. There was a relationship between the dose of anti-DAF $F(ab')_2$ administered and the intensity of staining for sheep $F(ab')_2$ in glomeruli (Table 1). In contrast, although the intensity of staining for intrinsic DAF was less than control rats, there did not appear to be a relationship between immunoreactive DAF remaining after Ab injection and the dose of the Ab. Figure 5 shows a glomerulus from a rat injected with the highest anti-DAF dose doubly stained for DAF and sheep $F(ab')_2$. Despite binding of function neutralizing Abs to DAF, and apparent reduction in DAF amount, there was no functional consequence apparent, either as renal insufficiency, albuminuria, or C3 deposition by IF microscopy (Table 1). Renal histology was similarly normal (not shown). Although altered DAF function has the potential to result in hemolysis [41, 42], there was no evidence for hemolysis in anti-DAF injected rats (Table 1). These studies showed that alternative pathway activation did not occur in vivo when the function of DAF was inhibited, results comparable to the preceding studies in vitro (as illustrated in Fig. 4).

The question remained whether DAF protected against classical pathway activation. Therefore, studies were done in which GEC-reactive, complement-fixing Abs (anti-megalin IgG2a monoclonal Abs) were present on the GEC surface, following which the function of DAF was neutralized with anti-DAF $F(ab')_2$ Abs. Although monoclonal anti-megalin Abs alone result in a small amount of detectable complement activation on GEC, there is not abnormal proteinuria [31]. To confirm these findings in this study, a control group consisted of animals treated identically, except pre-immune sheep $F(ab')_2$ was substituted for anti-DAF $F(ab')_2$. Given the preceding studies in which combined neutralization of DAF and CD59 was necessary to lead to complement activation in cultured GEC, a third group received anti-DAF and anti-CD59 $F(ab')_2$ together. Pathologic albuminuria was not found in any group (Table 2). Despite inhibition of a potentially

Table 1. Renal functional and morphologic data in rats injected with anti-DAF F(ab′)$_2$ Abs

Anti-DAF dose *mg*	BUN *mg/dL*	Hematocrit *%*	Albuminuria *μg/mg creatinine*	Rat DAF *IF score*	Sheep F(ab′)$_2$ *IF score*	Rat C3[a] *IF score*
0	ND	ND	ND	3.3	0	0
10	21.6	48.0	16.5	1.3	0.5	0
20	17.5	49.5	15.0	1.3	0.8	0
40	23.8	48.0	12.0	1.5	2.0	0

Normal rats were injected with varying doses of anti-DAF F(ab′)$_2$, following which urine was collected and animals were then sacrificed for blood and renal tissue collection. Data provided are the average of two animals studied in each group. ND is not done. Normal values are BUN <25 mg/dL, hematocrit 45–50%, and albuminuria <25 μg/mg creatinine.

[a] Staining was performed for both C3 and C3d

functional regulator of C3 activation, no difference in C3 staining, either as C3c or C3d, was apparent (Table 2).

A final group of studies addressed the question whether DAF was important to limit complement activation through the alternative pathway. To create conditions in which alternative pathway complement activation could occur, the PAN nephrosis model was used, in which there is substantial protein passage across the glomerular capillary wall. Thus, all components of the alternative pathway would be accessible to GEC in this model. Within six days of PAN injection, all animals had marked proteinuria (Table 3) and a moderate degree of renal insufficiency (Fig. 6). At this time, there was a reduction in immunoreactive DAF staining (IF score = 1.7 ± 0.1, compared to >3.0 in normal rats as shown in Fig. 1). While neutralization of DAF function by injection of anti-DAF Abs did not affect proteinuria (Table 3), it did slow the recovery from renal failure (Fig. 6). By day 11, all animals in the pre-immune IgG group had recovered normal renal function (BUN ≤30 mg/dL), while each animal in the anti-DAF group had persistent renal insufficiency (BUN >40 mg/dL). Intrinsic DAF and injected anti-DAF colocalized with a somewhat granular appearance (Fig. 7). In all animals with PAN, there was marked tubular complement deposition and only mild histological abnormalities (Fig. 8) that were not different between the two groups (Table 3). There was only a mild extent of glomerular C3 deposition prior to and after PAN injection (Fig. 8A). Even one day after anti-DAF injection, there was little C3 deposition (scores = 0.5 and 1.0 in the two animals biopsied on day 9). Hence, intact DAF function does appear to play a role in the recovery from acute renal failure occurring in the PAN nephrosis model.

Table 2. Measures of complement activation in animals with planted anti-megalin antibodies (Abs) in which complement regulators were inhibited

F(ab′)$_2$ Abs *injected on day 5*	Albuminuria *μg/mg creatinine*	C3 *IF score*	C3d *IF score*
Preimmune	9.5 ± 1.8	0.6 ± 0.1	1.0 ± 0.2
Anti-DAF	12.3 ± 2.8	0.6 ± 0.1	1.0 ± 0.2
Anti-DAF and anti-CD59	14.3 ± 3.1	0.5 ± 0.1	0.9 ± 0.1

Normal rats were injected with 15 mg each of K57/27 and K35/9 monoclonal anti-rat megalin Abs. Five days later, they received 20 mg of the indicated F(ab′)$_2$ Abs. The following day, urine was collected for measurement of albuminuria, and then animals were sacrificed and renal tissue stained for C3 and C3d.

DISCUSSION

Decay activating factor was originally isolated by Nicholson-Weller et al from guinea pig and human erythrocytes [43, 44]. After its characterization, it became clear that there was a gene family of complement regulatory proteins to which DAF belonged, termed the regulators of complement activation gene cluster [45]. In humans, the other members of this family are CR1, CR2, membrane cofactor protein, factor H and C4 binding protein, all located on band q32 of human chromosome 1 [46]. Each protein is characterized by the presence of ~60 amino acid short consensus repeats, which contain a framework of four invariant cysteines forming two disulfide bonds within each short consensus repeat [5, 46]. A characteristic in common among these proteins is their activity to inhibit complement C3/C5 convertases, which is due to an affinity for C3b and/or C4b in these convertases.

Soon after these human complement regulatory proteins were defined, homologous and analogous rodent genes were described. In the mouse, a similar gene stretch containing the regulators of complement activation family members is also on chromosome 1 [47, 48]. There are some interesting differences between humans and mice, including the origin of CR1 and CR2 from the same gene via alternative splicing, rather than from different genes as in humans [49, 50]. Work by Weis et al identified a mouse gene product called Crry because of its similarity to human CR1 [51]. In rats, work in the Okada lab identified a protein reactive with monoclonal antibody 5I2, which turned out to be rat Crry [52]. Because Crry has the combined activities of DAF and membrane cofactor protein, and neither of these two had been identified in rodents, Crry was considered to be their functional analog [6, 7]. Recently, however, the rodent homologs to DAF and membrane cofactor protein have been isolated and characterized [30, 48, 53–55].

In this study, we characterized rat DAF in kidney. By

Table 3. Disease outcomes in animals with PAN nephrosis given anti-DAF

Injection Day 8	Proteinuria *mg/day*		C3—glomeruli		C3—tubules		Histology—day 11	
	Day 5	Day 9	Day 8	Day 11	Day 8	Day 11	*% sclerosis*	*TI score*
Preimmune	142.6 ± 22.1	130.0 ± 23.2	1.0 ± 0.0	1.4 ± 0.1	3.5 ± 0.0	3.5 ± 0.0	3.1 ± 1.1	2.1 ± 0.3
Anti-DAF	126.8 ± 18.6	94.0 ± 19.2	0.9 ± 0.1	1.3 ± 0.1	3.1 ± 0.4	3.3 ± 0.4	3.4 ± 1.9	1.4 ± 0.9

Normal rats were given a single PAN injection on day 0. On day 8, animals were injected with either preimmune or anti-DAF IgG. The indicated disease variables were measured prior to (day 5 or 8) and after (day 9 or 11) antibody injection. A semiquantitative score of C3 staining in tubules and in glomeruli is provided. Histological assessment of the percent of glomerulosclerosis and a composite tubular injury (TI) score were derived as described in the **Methods** section. There were no statistical differences between preimmune and anti-DAF–injected groups in any of the measured variables.

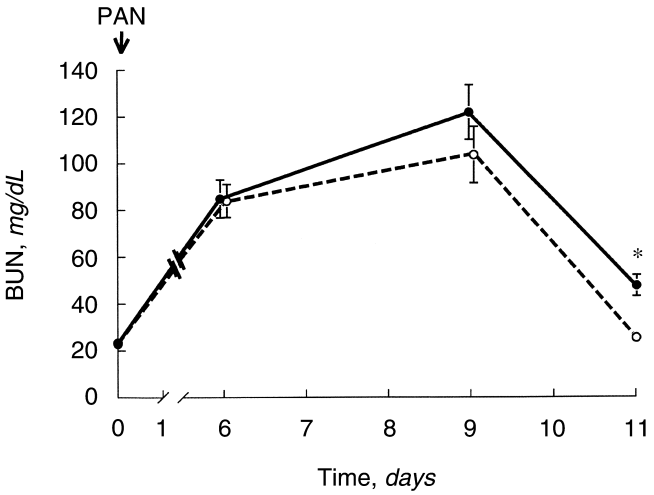

Fig. 6. Time course of renal function as measured by blood urea nitrogen (BUN) in puromycin aminonucleoside (PAN) nephrosis. Animals were given a single injection of PAN on day 0. On day 8 following PAN injection, animals either received anti-DAF ($N = 4$, ●) or preimmune IgG ($N = 5$, ○). Data shown are mean ± SEM. Where not shown, the error bar falls within the point. $^*P < 0.02$ versus pre-immune.

IF microscopy, using either monoclonal or polyclonal Abs, DAF was strongly present in glomeruli in a pattern consistent with GEC expression. That this was the case was confirmed with the higher resolution technique of immunoelectron microscopy, which showed that DAF was extensively and exclusively present on GEC. This pattern was unexpected, given the widespread distribution of DAF in other rat organs, including in many vascular endothelia [29]. GEC in culture also contained functional DAF. As expected from the known functions of human and rat DAF [46, 56], rat GEC DAF protected against both alternative and classical pathway activation. Like human erythrocyte DAF, this protein on GEC is linked via a glyosylphosphatidyl insitol (GPI) anchor [25, 57].

Our previous studies showed that DAF was present in cultured human GEC on which it was functionally active, and that immunoreactive DAF was present in human glomeruli [25]. At that time, there was some discrepancy over the distribution of human DAF in kidney [58, 59]. However, it is now clear DAF is expressed in human glomeruli predominantly in the juxtaglomerular apparatus, with lesser expression in cells of the glomerular capillary [23]. In disease states, there appears to be up-regulated expression in glomeruli [59–61], perhaps because of signals transduced directly by the complement activation [62].

While the study of DAF in human glomeruli is largely limited to its expression patterns in normal and diseased glomeruli, its role in rodents can be much more thoroughly evaluated. A widely used means of studying the role of a protein is by inducing its deficiency through targeted gene deletions. Both the Song and Medof laboratories have deleted the gene for the GPI-linked form of mouse DAF [42, 63]. These mice are phenotypically normal, with no evidence for abnormal complement activation, consistent with the redundancy of complement regulators. However, DAF-deficient mice had increased susceptibility to complement activation and disease manifestations in two variants of the nephrotoxic serum nephritis model [64, 65]. Mice have prominent glomerular expression of DAF by IF microscopy [63], which is comparable to what we have shown here in rats. Furthermore, DAF-deficient animals with nephrotoxic serum nephritis developed GEC morphological abnormalities [65] consistent with DAF having a functional role on GEC in mice.

Because of the apparent high expression of DAF on rat GEC, and given our data on cultured rat GEC on which DAF limited complement activation, it seemed likely that DAF present on GEC in vivo would be functionally active. To examine this hypothesis, we utilized polyclonal anti-DAF Abs that neutralized the function of DAF in vitro. Upon IV injection into normal rats, anti-DAF $F(ab')_2$ Abs had ready access to GEC DAF, with both binding of Abs and apparent loss of DAF possibly through antigenic modulation of the GPI-linked DAF [66]. Despite this loss of immunoreactive DAF protein and binding by inhibitory Abs, there was no evidence that complement was activated, nor was the GEC altered, as evidenced by normal morphology and urinary albumin excretion. This indicates that GEC complement regulation is unnecessary given its unique location beyond the glomerular barrier to protein passage, and/or, there are other complement regulators, such as Crry,

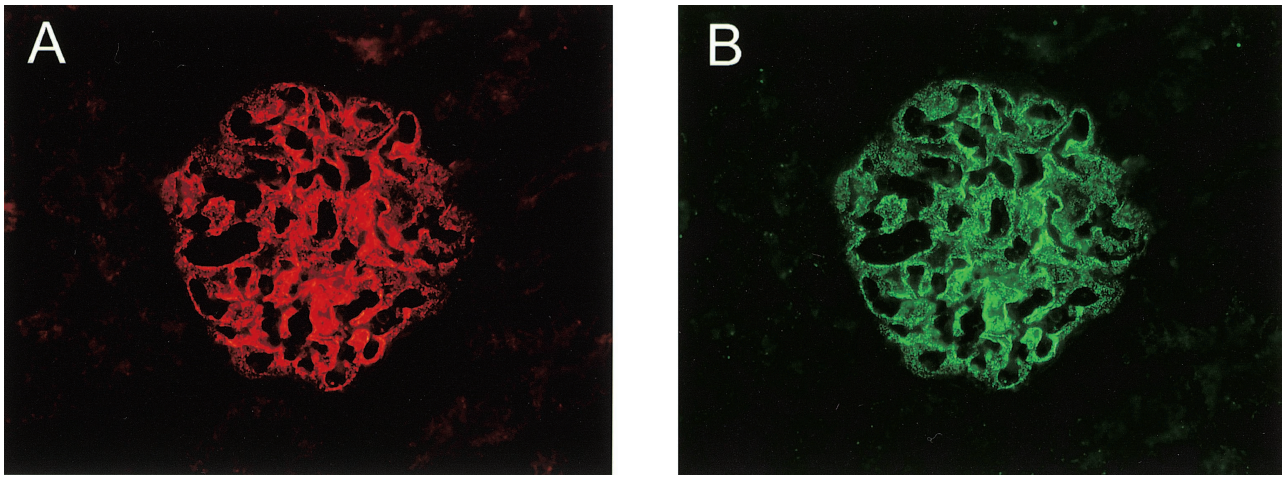

Fig. 7. Immunofluorescence staining for rat DAF (*A*) and sheep IgG (*B*) following injection of sheep anti-rat DAF in PAN nephrosis. Shown is IF from a single animal 9 days after PAN administration and 1 day after anti-DAF injection. The same dual labeling technique as described in the legend to Fig. 5 was utilized to detect intrinsic DAF and glomerular bound sheep anti-DAF.

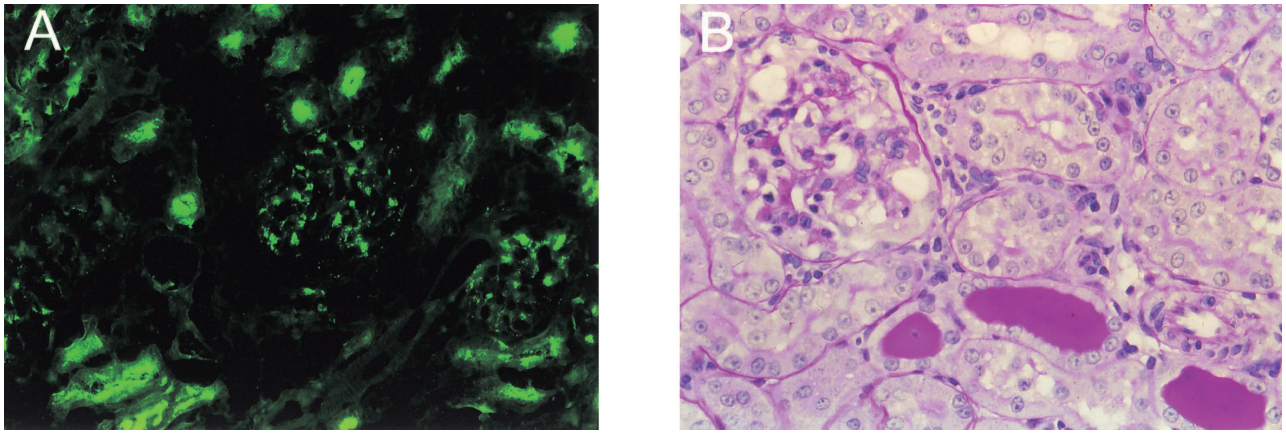

Fig. 8. Immunofluorescence staining for C3 (*A*) and histological features (*B*) in animals with PAN nephrosis given anti-DAF or pre-immune IgG. There was intense staining for C3 primarily in the luminal aspects of proximal tubules in all animals. A glomerulus in the center shows a modest extent of mesangial C3 staining that was typical for animals, with no difference apparent between those given anti-DAF and pre-immune IgG (Table 3). (B) Examples of glomerular and tubular histological abnormalities. The glomerulus has segmental hyalinosis and GEC swelling, while three tubular segments contain casts. Although several other histological features were seen, these occurred at the highest frequency.

which are in place to limit complement activation at the level of C3 and C5 convertases [27].

Because of these negative results, combined with observations in cultured cells that Ab-directed complement activation was considerably more effective than spontaneous (alternative pathway) complement activation, we activated complement on GEC with anti-megalin Abs. Thus, in the setting of anti-megalin Abs, a small amount of C3c and C3d was detected in glomeruli by IF microscopy, consistent with ongoing complement activation [40]. The possibility that DAF might limit complement activation by these anti-megalin Abs was investigated by injecting neutralizing Abs. Yet, in this setting, there was no difference in C3 staining intensity by IF microscopy. Since differences in C3 staining intensity do not always reflect productive complement activation leading to generation of C5b-9, we utilized anti-CD59 Abs in combination with anti-DAF Abs. Not surprisingly, there was no difference in C3 staining intensity, as inhibiting CD59 would not be expected to affect C3 convertases. However, in the setting of anti-megalin Abs and neutralization of DAF and CD59 function, there was no increase in albuminuria above normal. These findings are different than in our earlier study in which Crry and CD59 were inhibited, in which case, proteinuria was increased [31]. Taken together, these findings could indicate either that Crry is the functional C3/C5 convertase regulator, or that the GEC has site-specific complement regulation, with Crry principally on the soles and DAF on the apical surfaces, corresponding to their locations in vivo.

It is important to point out the limitations of our studies examining the role of DAF in vivo. For these, Abs were injected that inhibited the function of DAF in vitro; such an experimental strategy is commonly used [31, 67]. In practical terms, this was the only approach available as we lacked an inhibitory pharmacologic agent or rats that were genetically deficient in DAF. One important issue is how much of the injected anti-DAF $F(ab')_2$ Abs actually bound to their designated target on the GEC. With the use of large quantities of injected Abs (up to 40 mg), significant binding to glomeruli was apparent soon after injection. With this, immunoreactive DAF declined, either due to antigenic modulation and/or to epitope masking by the bound anti-DAF Abs. However, the fact that DAF has a widespread distribution in the rat including on endothelia and blood cells [29] means that the injected Abs had many targets, and it is likely only a minority of that injected was available for glomerular binding. Furthermore, the ~90 kD $F(ab')_2$ molecule would have limited access across the glomerular basement membrane and GEC slit diaphragm to access the apical surface of GEC. Given these considerations, it is likely that DAF was not inhibited in its entirety in these studies, and we must be cautious in our interpretations that DAF does not play any role in complement regulation in vivo.

Under physiological circumstances, or even those in which anti-megalin Abs are present, there is relatively little protein accessibility to the GEC. In our studies, such relevant proteins include both the activating complement proteins and the neutralizing anti-DAF Abs. Therefore, the PAN nephrosis model was used as a model in which there is marked protein passage across the glomerular capillary wall. At the time of injection of anti-DAF (or pre-immune control) Abs, animals had heavy proteinuria and renal insufficiency, the latter attributable, at least in part, to complement activation on the tubules [39, 68]. Neutralization of DAF function had no effect on proteinuria, glomerular or tubular complement deposition or histological features of disease. However, animals in which DAF function was neutralized did not recover normal renal function by day 11 of the model compared to those animals receiving pre-immune IgG. The renal failure in this model is interesting, and may well be attributable to complement activation on the renal tubules [39, 68]. Consistent with this was the presence of a marked degree of complement activation on tubules and a modest degree of histological damage. Not surprisingly, neutralization of DAF function affected neither of these, as our current study showed DAF to be absent from tubular cells, while the complement regulator Crry has a protective role in this model [68]. It is interesting that while anti-DAF clearly altered glomerular filtration, it did not affect histological measures of glomerular disease in this model, those being the extent of glomerulosclerosis and C3 deposition. Notably, in contrast to the prominent tubular deposition of C3, glomerular C3 staining was relatively modest at all times in the disease course. Based upon our data, it does seem likely that DAF on GEC does hasten the recovery from the renal failure that occurs in this model, and thus complement activation on these cells is of pathogenic significance in PAN nephrosis.

Rat glomerular DAF is predicted to be anchored to the GEC plasma membrane through a GPI linkage [25]. Similarly, CD59 has a GPI anchor [69]. The GEC has an interesting polarization with the apical surface free in the urinary space, while the basal surface is opposed to the glomerular basement membrane. Such a polarization of GPI-linked proteins to apical surfaces has been reported for many epithelial cells and, therefore, GEC appear to follow this general rule applicable to epithelial cells [70, 71].

In summary, our study characterizes DAF present on rat GEC. In GEC in vitro, DAF is an effective complement regulator, and works in concert with CD59 to limit complement activation. In GEC in vivo, DAF is present extensively and exclusively on the apical surfaces of GEC. Yet, it may not be essential to prevent complement activation from occurring under normal circumstances and in those cases in which complement-activating Abs are present on the basal surfaces of GEC in vivo. However, in proteinuric conditions, DAF appears to protect GEC from spontaneous complement activation from the filtered complement proteins. Whether DAF also has a role completely removed from its complement regulatory activity, such as transducing signals to GEC [56], remains to be determined.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants DK34972 (DRA) and DK41873 (RJQ), and by the Wellcome Trust Senior Clinical Fellowship (BPM). Drs. Bao, Cunningham and Doshi were supported by National Institutes of Health Training Grant DK07510. We thank Dr. Donna Mendrick for supplying the anti-megalin Abs and Randy Anderson for expert technical assistance.

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