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Respective Roles of Decay-Accelerating Factor and CD59 in Circumventing Glomerular Injury in Acute Nephroptic Serum Nephritis

Feng Lin,* David J. Salant,‡ Howard Meyerson,* Steven Emancipator,* B. Paul Morgan,† and M. Edward Medof**

Decay-accelerating factor (DAF or CD55) and CD59 are regulators that protect self cells from C3b deposition and C5b-9 assembly on their surfaces. Their relative roles in protecting glomeruli in immune-mediated renal diseases in vivo are unknown. We induced nephroptic serum (NTS) nephritis in Daf1−/−, CD59a−/−, Daf1−/−CD59a−/−, and wild-type (WT) mice by administering NTS IgG. After 18 h, we assessed proteinuria, and performed histological, immunohistochemical, and electron microscopic analyses of kidneys. Twenty-four mice in each group were studied. Baseline albuminuria in the Daf1−/−, CD59a−/−, and Daf1−/−CD59a−/− mice was 82, 83, and 139 as compared with 92 μg/mg creatinine in the WT controls (p > 0.1). After NTS, albuminuria in CD59a−/− and WT mice (186 ± 154 and 183 ± 137 μg/mg creatinine, p > 0.1) was similar. In contrast, Daf1−/− mice developed severe albuminuria (378 ± 520, p < 0.05) that was further exacerbated in Daf1−/−CD59a−/− mice (577 ± 785 μg/mg creatinine, p < 0.05). Glomerular histology showed essentially no infiltrating leukocytes in any group. In contrast, electron microscopy revealed prominent podocyte foot process effacement in Daf1−/− mice with more widespread and severe damage in the double knockouts compared with only mild focal changes in CD59a−/− or WT mice. In all animals, deposition of administered (sheep) NTS Ig was equivalent. This contrasted with marked deposition of both C3 and C9 in Daf1−/−CD59a−/− and Daf1−/− mice, which was evident as early as 2 h post-NTS injection. The results support the proposition that in autoantibody-mediated nephritis, DAF serves as the primary barrier to classical pathway-mediated injury, while CD59 limits consequent C5b-9-mediated cell damage. The Journal of Immunology, 2004, 172: 2636–2642.

Becare the binding of opsonic C3b fragments and lytic C5b-9 complexes to biological membranes is indiscriminate and can occur on self cells as well as foreign targets, self cells must be protected from injury that could result from activation of the cascade on their surfaces (reviewed in Refs. 1 and 2). Three complement regulators, decay-accelerating factor (DAF)3 or CD55, membrane cofactor protein (MCP or CD46), and CD59 function intrinsically in the membranes of self cells to provide this protection (3–5). They are expressed ubiquitously on the surfaces of all cells that can come into contact with complement (6–8). DAF inactivates any C3 (C4b2a and C3bBb) and C5 (C4b2a3b and C3bBb3b) convertases that assemble on self cells by accelerating the decay of these enzymes (9, 10). MCP serves as a cofactor for the cleavage of (nonconvertase-associated) cell-bound C4b and C3b by the serum protease factor I (11). CD59 interferes with the uptake of C9 by cell-bound C5b-8 and subsequent insertion/polymerization of C9, thus preventing the formation of lytic C5b-9 membrane attack complexes (MAC) that bring about lysis (12, 13). In patients with the hemolytic disorder paroxymal nocturnal hemoglobinuria, affected blood cells that lack DAF and CD59 exhibit heightened uptake of C3b on their surfaces in vivo (14, 15) and undergo lysis intravascularly, documenting the physiological importance of these two regulators (16, 17).

Recently, for purposes of investigating the respective protective roles of DAF and CD59 activities in vivo in various disease states, DAF (18, 19) and CD59 (20, 21) knockout mice have been prepared. Differently from humans, mice have two DAF (Daf1 and Daf2) (22) and two CD59 (CD59a and CD59b) genes (23, 24). The Daf1 gene product is predominantly GPI anchored and widely expressed on all tissues (22), while the Daf2 gene product is predominantly transmembrane anchored (22) and is constitutively expressed only in testis (18, 25) and splenic dendritic cells (18). A similar pattern of expression pertains for the two CD59 genes (26). Although mice have an MCP gene, its expression is limited to testis, like the Daf2 and CD59b genes. Systemically in its place, they express another regulator termed complement receptor-related protein Y (Crry), but knockout of this gene can only be achieved on an alternative pathway deficient (C3−−−) background (see Discussion). Thus, for the knockouts, the Daf1 and CD59a genes, considered to be counterparts of DAF and CD59 genes in humans, were targeted.

Nephroptic serum (NTS)-induced nephritis is a widely used model of Ab-induced glomerular disease (reviewed in Ref. 27). In the passive heterologous form of the disorder using low doses of administered complement-fixing Ab, renal injury evidenced by albuminuria occurs in the first 18 h and is almost entirely complement dependent (28–31). Analyses of the complement

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3 Abbreviations used in this paper: DAF, decay-accelerating factor; Crry, complement receptor-related protein Y; EM, electron microscopy; MAC, membrane attack complex; MCP, membrane cofactor protein; NTS, nephroptic serum; WT, wild type; DKO, double knockout.
involvement in this and other animal models of Ab-mediated glomerular disease have implicated terminal pathway components in the renal damage (32–38).

In a previous study (39), we showed that when given low doses of NTS, Daf1<sup>−/−</sup> mice suffer more profound proteinuria, markedly more C3b deposition in their glomeruli, and much more severe podocyte damage compared with wild-type (WT) controls. The pathological changes are Ab and complement dependent, as no evidence of leukocyte involvement is evident. In view of previous studies implicating the MAC in glomerular injury in this model, an understanding of the relative roles of DAF and CD59 in circumventing complement-mediated injury to glomerular cells is important. To address this question, in the present study, we took advantage of the availability of Daf1<sup>−/−</sup> mice, CD59<sup>a−/−</sup> mice, and Daf1<sup>−/−</sup>CD59<sup>a−/−</sup> double knockout mice, and compared the changes that occur in each knockout with those that occur in WT controls.

Materials and Methods

Animals and Abs

Daf1 knockout mice were prepared, as previously described (18). Briefly, murine GK129 embryonic stem cells were used and knockout was achieved by Cre/LoxP-mediated deletion. CD59<sup>a−/−</sup> mice were generated by replacing exon 3 of the CD59 gene with Neo using 6 kb of homologous sequence (20). In both cases, chimeric mice were bred four generations with the C57BL/6 strain. Daf1<sup>−/−</sup>CD59<sup>a−/−</sup> double knockout mice were prepared by breeding Daf1<sup>−/−</sup> and CD59<sup>a−/−</sup> mice with each other. Single and double knockouts were typed by flow cytometric analyses of their erythrocytes (E<sup>+</sup>) following staining with anti-DAF mAb 2C6 (40) and anti-CD59 mAb MEL-4 (41). The mice were maintained in the Animal Resource Center of Case Western Reserve University, and experiments were performed according to an approved protocol of the Institutional Animal Care and Use Committee. All mice were studied at 8–10 wk of age.

The γ1 fraction of IgG was purified (by DEAE-Sephadex chromatography) from NTS raised by immunizing sheep with rat glomeruli (39). An i.v. administered dose providing for injury to mouse glomeruli that is maximally complement dependent (30, 31) was used. FITC-conjugated goat anti-mouse C3 Ab (1/5000) or FITC-conjugated rabbit anti-sheep IgG Ab were obtained from Zymed Laboratories (San Francisco, CA), and FITC-conjugated rabbit anti-mouse DAF mAb 2C6 (40) or anti-CD59 mAb MEL-4 (41). The mice were maintained in the Animal Resource Center of Case Western Reserve University, and experiments were performed according to an approved protocol of the Institutional Animal Care and Use Committee. All mice were studied at 8–10 wk of age.

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Induction of nephritis

Glomerulonephritis was induced, as described previously (39). Briefly, 500 μg of NTS was injected into the tail vein, and mice were placed in metabolic cages. Urine samples collected after 18 h were analyzed for creatinine and albumin concentrations on a Hitachi/Roche 917 autoanalyzer (Hitachi Roche Diagnostics, Mannheim, Germany). The amount of excreted albumin was normalized for the amount of excreted creatinine, i.e., μg albumin/mg creatinine. All averages are given as means.

Immunofluorescence and histological staining

At the time of urine collection, i.e., 18 h after NTS injection (in some cases 2 h), kidneys from mice were harvested. For immunofluorescence staining, kidney samples were snap frozen in liquid nitrogen and cut at (5 μm) on a cryostat. Cryostat sections were labeled with either FITC-conjugated goat anti-mouse C3 Ab (1/5000) or FITC-conjugated rabbit anti-sheep IgG Ab (1/1000) or with the same concentrations of FITC-conjugated goat IgG or FITC-conjugated rabbit IgG as controls. For detection of deposited C9, sections were blocked with goat Ig and stained with 1/600 dilution of rabbit anti-rat C9 antiserum or with the same dilution of normal rabbit serum. After washing, slides were incubated with FITC-labeled goat anti-rabbit IgG. In all cases, stained sections were examined with an Olympus OM6 fluorescence microscope.

Alternative samples of the same kidneys were fixed in 10% buffered Formalin, embedded in Tissue Prep (Fisher Scientific, Fair Lawn, NJ), and sectioned at 5 μm. Sections were stained with H&E, followed by periodic acid Schiff reagent, and examined with an Olympus BH 2 microscope.

Electron microscopy (EM)

Samples of kidneys were fixed in 2.0% glutaraldehyde and 0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide, and embedded in Spurr’s epoxy. Ultrathin sections were picked up on nickel grids, stained with uranyl acetate/lead citrate, and examined with a JEOL 101C microscope. At least six (×6700) fields from each of at least four glomeruli per mouse were photographed.

FACS analyses

One million E<sup>+</sup> from Daf1<sup>−/−</sup>, CD59<sup>a−/−</sup>, and Daf1<sup>−/−</sup>CD59<sup>a−/−</sup> mice or their WT littermates were incubated for 30 min on ice with 10 μg/ml rat anti-mouse DAF mAb 2C6, 10 μg/ml rat anti-mouse Crry mAb 5D5 (43), or 10 μg/ml rat anti-mouse CD59 mAb MEL-4, and with the same concentrations of their corresponding nonrelevant controls. Following washing, the cells were secondarily incubated for 30 min on ice with 5 μg/ml FITC-labeled anti-rat IgG, and, after washing, the stained cells were analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

PCR analyses

Total RNA from kidneys of WT and Daf1 knockout mice was extracted using TRIzol (Life Technology, Rockville, MD). For semi-quantitative PCR, identical amounts of total RNA were used to synthesize cDNAs using an oligo(dT) primer for reverse transcription, and PCR was performed for 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s using the synthesized cDNAs as templates. Mouse Crry cDNA was amplified with primer P<sub>1</sub> (5′-GAGAAATGGTCTGACTGC-3′) and P<sub>2</sub> (5′-GTCTACAGACAGCATGACTGCA-3′); mouse CD59a cDNA was amplified with primer P<sub>3</sub> (5′-GCTTCGCGGTGTTGTTGACC-3′) and P<sub>4</sub> (5′-GAGAAATGGTCTGACTGC-3′).
(5'-ATGGCACCAGAACCAGGTCC-3'). CD59a cDNA was amplified with primers P6 (5'-GAGGGGCTCATCTTCTCC-3') and P8 (5'-AAAGAGGTCTTCCTGGGTG-3'). Mouse actin cDNA was amplified concurrently as a control using primers P4 (5'-CACGGCAGCCGACTTCC-3') and P5 (5'-TGGGCTTTGCGGTGGGG-3'). Daf1 and Daf2 cDNAs were amplified using primers P6 (5'-ATGATCGTGCGGCGGCCCT-3'), P7 (5'-CTCAGAAACTCATCGTCCCA-3'), and P10 (5'-TGATTTTCTTGAGGTAGGTGTT-3').

Results
As a first indicator of the in vivo importance of DAF and CD59 activities in protecting glomeruli following NTS administration, urines were collected from Daf1−/−, CD59a−/−, Daf1−/−CD59a−/−, and WT mice 18 h after injection of the Ab, and proteinuria was quantitated. Six independent experiments with four animals in each group were conducted, and the data were combined into one set. Baseline albuminuria in the Daf1−/−, CD59a−/−, and Daf1−/−CD59a−/− mice was 82, 83, and 139 as compared with 92 μg/mg creatinine in the WT controls (p > 0.1). As shown in Fig. 1, compared with WT controls, the NTS caused markedly increased proteinuria in Daf1−/− mice (378 ± 520 vs 186 ± 154 μg albumin/mg creatinine, p < 0.05), as previously reported (39). Unexpectedly, it did not result in a significant difference between the CD59a−/− and WT groups (186 ± 154 vs 183 ± 137 μg albumin/mg creatinine, p = 0.45). In contrast, the injection caused ~1.6-fold higher proteinuria levels (577 ± 783 μg albumin/mg creatinine) in the Daf1−/−CD59a−/− double knockout mice compared with the selective Daf1 knockouts (p < 0.05).

To compare the pathological changes in glomeruli of the four mouse groups, immunofluorescence, histological, and EM analyses of kidneys were performed. Three replicate experiments were done. As shown in Fig. 2A for representative specimens at the (above) 18-h time point, immunofluorescent staining revealed markedly increased C3b deposition in glomeruli of both Daf1−/− and Daf1−/−CD59a−/− mice, which was similar in intensity, compared with little or no deposition in the WT controls. In accordance with the proteinuria measurements, minimal deposition of C3b was evident in the CD59a−/− mice as in the WT mice, consistent with normal DAF function. Similar to the C3 staining, staining for deposited C9, as shown in Fig. 2B, revealed markedly increased amounts of the protein not only in the Daf1−/−CD59a−/− double knockouts, but also in the selective Daf1 knockouts, albeit in lesser quantities. As with the C3 staining, minimal C9 staining was evident in the CD59a−/− mice similar to that in the WT controls. In glomeruli of all of the groups, staining for the administered NTS IgG showed equivalent intensity and distribution of deposited sheep Ig (Fig. 2C). There was no C3, C9, or sheep Ig staining in glomeruli of any of the knockouts or in WT mice that were not given NTS.

EM analyses at 18 h of representative glomeruli for each group are given in Fig. 3. Daf1−/− mice, as previously found (39), showed effacement and diffuse flattening of podocyte foot processes, whereas Daf1−/−CD59a−/− double knockouts showed more severe damage to epithelial cell architecture and structure. In contrast, CD59a−/− knockouts, similar to the WT mice, showed only minimal segmental changes. Characteristic of the heterologous phase of the model, there were no electron-dense deposits. There also was no evidence of leukocyte margination.

The comparative histologic appearance of representative glomeruli from each group of mice is shown in Fig. 4. As seen, no gross changes were visible in any of the groups. Immunoperoxidase staining for mouse neutrophils at 18 h showed no positive cells in glomeruli or in tubular interstitium. Moreover, at 18 h, counting of cells in 10 glomeruli from each animal showed <1 leukocyte per glomerulus in any of the Daf1−/−, CD59a−/−, Daf1−/−CD59a−/− mice or the WT controls.

To determine how rapidly the complement deposition and glomerular damage occur in the disease, kidneys were examined 2 h post-NTS injection. As seen in Fig. 5A, both C3 and C9 deposition...
in the $Daf1^{+/−}$ and $Daf1^{+/−}CD59a^{−/−}$ double knockouts was clearly evident, although less intense than at 18 h. As at 18 h, no deposition of either component at 2 h was observed (data not shown) in the WT and $CD59a^{−/−}$ mice. As seen in Fig. 5B, substantial podocyte injury in the two knockouts, although less severe, also was already clearly present.

FACS analyses of E<sub>mo</sub> from each knockout verified that, in each case, normal expression levels of all nontargeted regulatory proteins were retained, i.e., Crry, a second C3 convertase regulator (see Discussion) and CD59 in the case of $Daf1^{+/−}$ mice, DAF and Crry in the case of $CD59a^{−/−}$ mice, and Crry in the case of $Daf1^{+/−}CD59a^{−/−}$ double knockouts. To confirm that the levels of the nontargeted complement regulators in kidney tissue likewise were not altered, semiquantitative PCR using RNA extracted from kidneys of the animals was performed. PCR additionally was performed to confirm that there was no compensatory expression of DAF or CD59 protein deriving from the $Daf2$ and $CD59b$ genes. As seen in Fig. 6, these analyses, as with flow cytometry, showed no significant alterations in nontargeted regulators, and no compensatory change in minimally detectable $Daf2$- or $CD59b$-derived message (data not shown).

**Discussion**

We and others previously demonstrated that complement activation in the glomeruli of mice with NTS-induced nephritis is tightly regulated at the level of C3, such that there is only minimal complement-mediated injury as long as DAF and Crry are present (28–30, 39). In our previous studies in DAF-deficient mice (39), we found markedly increased pathology. The mechanism of glomeruli injury and the extent to which pathology would be increased in the absence of CD59, which protects against autologous C5b-9 assembly, however, remained unclear. In the present study, we used $Daf1^{+/−}$, $CD59a^{−/−}$, and $Daf1^{+/−}CD59a^{−/−}$ double knockouts to characterize the respective roles of the two regulators. By all criteria, we found that $Daf1^{+/−}CD59a^{−/−}$ double knockouts suffered the greatest damage. These mice showed the highest proteinuria level, greatest C3 and C9 deposition in glomeruli, and most extensive podocyte damage by EM, the collective results thus documenting that both regulators are critical in protection. Surprisingly, we did not detect a significant difference between selective $CD59a$ knockouts and WT controls. There was no significant increase in proteinuria and no significant difference in epithelial cell damage.

**FIGURE 3.** EM examination of glomeruli 18 h after NTS treatment of WT mice (A) shows generally intact epithelial podocytes, with only segmental flattening and fusion. Glomeruli from $CD59a^{−/−}$ mice show a similar pattern (C). In contrast, epithelial podocytes from $Daf1^{+/−}$ mice (B) show extensive flattening and fusion and focal lipid vacuole formation, while podocytes from $Daf1^{+/−}CD59a^{−/−}$ (D) mice show the most severe damage (original magnification $×20,000$).

**FIGURE 4.** By light microscopy, after injection of NTS IgG, glomeruli from WT mice (A) appear entirely normal. Glomeruli from $Daf1^{+/−}$ (B), $CD59a^{−/−}$ (C), and $Daf1^{+/−}CD59a^{−/−}$ (D) mice do not show significant differences.
damage as discernible by EM. Our RT-PCR and FACS analyses verified that there were no compensatory changes in any of the regulators. Taken together, the findings indicate that while the importance of the activity of DAF is readily evident, the activity of CD59 becomes clearly apparent in the absence of DAF, and thus that the activities of both regulators are essential.

**FIGURE 5.** A, Immunofluorescent staining for mouse C3 and C9 in representative glomeruli from WT, CD59a−/−, Daf1−/−, and Daf1−/−CD59a−/− mice 2 h after injection with sheep NTS IgG. B, EM analyses of glomeruli from each group of mice show initial changes similar to, but less severe than, those apparent at 18 h (Fig. 3).

**FIGURE 6.** A, Semiquantitative RT-PCR analysis of Daf1, CD59, and Crry mRNA expression in representative kidneys from WT, Daf1−/−, CD59a−/−, and DKO mice before and 18 h after NTS injection. Mouse actin mRNAs were amplified as a control. No PCR product was observed for Daf2 either before or after NTS in any of the groups (data not shown). An identical low intensity PCR product for CD59b was observed in all of the groups that did not differ before or after NTS (data not shown). Systematic analyses using both polyclonal and monoclonal anti-CD59b Abs have shown no CD59b expression in the kidney (26). B, FACS analyses of erythrocytes from WT, Daf1−/−, CD59a−/−, and DKO mice using 2C6 anti-DAF, MEL-4 anti-CD59a, and 5D5 anti-Crry mAbs.
The findings that, in the absence of DAF regulation in NTS nephritis, CD59 deficiency leads to increased glomerular C9 and renders mice more susceptible to Ab-mediated injury strongly implicate the C5b-9 MAC in mediating glomerular injury. This is consistent with the observation that C6-deficient rabbits are partly protected from NTS-induced nephritis (35). Although this model has been regarded historically as a model of antilglomerular basement membrane nephritis, in addition to reactivity to type IV collagen, NTS identifies several podocyte cell surface Ags (44). These cell surface Ags serve as targets for nephritogenic Abs and lead to activation of the classical pathway, assembly of C5b-9, and complement-mediated cytotoxicity. In this regard, the mechanisms of injury, in principle, should be similar to other models of C5b-9-mediated glomerular cell injury (45), including experimental membranous nephropathy (32, 33, 37, 46) and renal microvascular injury that is induced by Ab to glomerular endothelial cells (38). Although our staining for C9 provides evidence that damage in all cases is, in fact, MAC mediated, the data, taken together, argue that DAF regulation is the principal barrier to activation of C5 and to subsequent cell surface assembly of C5b-8, the point at which CD59 exerts its activity.

The importance of intrinsic regulators in protecting self tissues from autologous complement-mediated injury is widely established. It is highlighted in two diseases. In the hemolytic disorder paroxysmal nocturnal hemoglobinuria (1), the absence of DAF and CD59 on affected blood elements renders them susceptible to heightened surface uptake of autologous C3b and intravascular lysis in vivo, eventuating not only in anemia, but, in a large proportion of patients, life-threatening or mortal thrombosis. Rare individuals with genetic deficiency of MCP constitute one subset of patients who develop the frequently fatal hemolytic uremic syndrome (47).

A number of studies have shown that Crry (48), the rodent analog of MASP, is important in protecting the kidney in NTS nephritis. Renal injury is diminished in Crry transgenics (29) or if Crry-Ig is administered to WT animals (30). Our findings in this study argue that Crry is unable to compensate for deficiency in DAF when complement-mediated damage is initiated by the classical pathway. In vitro and in vivo studies have provided evidence that Crry may be more important for controlling the alternative pathway (49, 50).

The mechanism of proteinuria in acute NTS nephritis is uncertain, but is most likely due to sublethal MAC-mediated podocyte injury with subsequent loss of the barrier function of the glomerular capillary wall. By analogy to experimental membranous nephropathy, the loss may eventuate from perturbations in intracellular calcium, activation of phospholipases and stress proteins, ATP depletion, and alterations in the actin cytoskeleton, all culminating in structural abnormalities of podocyte foot processes and possibly in disruption of slit diaphragms (46, 51–53).

A second widely studied form of NTS-induced nephritis is the autologous phase of the disease (reviewed in Ref. 54). In this model, animals are preimmunized with sheep Ig and after the elaboration of anti-sheep Ig Ab, NTS is administered. The pathology in this model is due largely to the influx of leukocytes and release from the cells of enzymes, cytokines, and other cellular mediators of inflammation. Studies of this latter phase of the disease, in other work, have yielded results similar to ours. In Daf1 knockout mice, there is markedly increased polymorphonuclear cell infiltration and massive glomerular destruction with crescent formation (55), most likely in large part resulting from the generation of chemotactic C5a anaphylatoxin (56). In CD59a−/− mice, about half of the animals suffer more profound kidney damage evidenced by proteinuria and EM, detectable damage to epithelial cells, but not increased polymorphonuclear influx (57), consistent with the expected absence (due to intact DAF activity) of an increase in C5 cleavage/C5a generation. Studies in the autologous model have not yet been performed with Daf1−/−CD59a−− double knockouts.

In summary, our studies, taken together with previous and recent work by others, highlight the importance of intrinsic regulators in the kidney, in particular glomerular epithelium, in protecting against complement-mediated damage in immune and inflammatory glomerulopathies. They show that DAF is critical in preventing the induction of the pathway and provide further evidence that injury in the acute model is mediated principally by the MAC.

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