CD59a Deficiency Exacerbates Ischemia-Reperfusion Injury in Mice

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The terminal complement components C5a and the membrane attack complex are involved in the pathogenesis of ischemia-reperfusion injury in many organs. CD59 is the major regulator of membrane attack complex formation. Mice deficient in the *Cd59a* gene (*mCd59a*^{-/-}) were used to investigate the role of CD59 in renal ischemia-reperfusion injury. Unilateral ischemia-reperfusion injury was induced by clamping the left renal pedicle for 30 minutes under general anesthetic. Mice were studied at 72 hours and 2 weeks after ischemia-reperfusion injury. *mCd59a*^{-/-} mice developed significantly greater tubular injury ($P = 0.01$), tubulointerstitial apoptosis ($P = 0.02$), and neutrophil influx ($P = 0.04$) than controls at 72 hours after ischemia-reperfusion. Two weeks after ischemia-reperfusion, *mCd59a*^{-/-} mice exhibited more severe tubular damage predominantly in a corticomedullary distribution than controls ($P = 0.02$). Quantification of interstitial leukocytes revealed significantly greater numbers of infiltrating lymphocytes (but not macrophages) in *mCd59a*^{-/-} mice than controls ($P = 0.04$) at 2 weeks. At both time points, significantly more C9 (as a marker of membrane attack complex) deposition occurred in a peritubular distribution in *mCd59a*^{-/-} mice than controls. In conclusion, these results demonstrate that the lack of CD59a, by allowing unregulated membrane attack complex deposition, exacerbates both the tubular injury and the interstitial leukocyte infiltrate after ischemia-reperfusion injury in mice. (*Am J Pathol* 2004, 165:825–832)

Ischemia-reperfusion injury (IRI) arises after blood flow restoration after a significant period of ischemia.¹ It is of major clinical importance because it is the predominant cause of tissue damage in conditions such as stroke,

myocardial infarction, cardiopulmonary bypass, and intestinal ischemia. Renal IRI is the primary cause of acute renal failure in both native kidneys and allografts and is of major importance in graft survival.² There is at present no specific therapy.

The pathogenesis of IRI is a complex and incompletely understood process that involves a series of events whose final common endpoint is apoptotic or necrotic cell death. During ischemia antigens such as P-selectin are expressed on the cell surface and ICAM-1 and E-selectin are up-regulated.^{3,4} Once reperfusion occurs, inflammatory mediators including leukocytes and complement are able to reach the site of tissue injury. This sets up a cascade of tissue injuries mediated by neutrophils, reactive oxygen species, and complement components. Activation of the complement system occurs through both the classical and alternative pathways, and leads to the production of chemotactic factors, such as C5a, and to the deposition of the MAC. The role of complement in IRI has been extensively investigated in a variety of organs including intestine, heart, lung, brain, muscle, and kidney. Inhibition of complement at the level of the C3 convertase in myocardial or intestinal IRI using soluble complement receptor type 1 significantly reduced tissue damage in rats.⁵ C6-deficient rabbits were protected from myocardial ischemia, both in terms of infarct size and neutrophil influx to the infarcted region, compared with C6-sufficient animals, suggesting that the MAC is more important than C5a in this model.⁶ Furthermore there is evidence, from a mouse model of renal IRI, that MAC deposition rather than C5a plays a predominant role in the pathophysiology of this type of injury.⁷ In this study it was also demonstrated that renal damage secondary to IRI was not initiated by the classical pathway of complement. In support of these data, Thurman and colleagues⁸ showed that absence of factor B, which renders the alternative pathway functionally inactive, ameliorated renal IRI. However, there is also evidence for an important contribution of C5a, in addition to MAC, in causing renal damage. Inhibiting the C5a receptor protected mice from renal IRI at 24 hours but did not prevent up-regulation of tumor necrosis factor- α production or cellular apoptosis,

Supported by the Wellcome Trust (grant 071467).

D.T. was a recipient of a fellowship from the National Kidney Research Fund.

Accepted for publication May 19, 2004.

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suggesting that both MAC and C5a are important in causing renal disease after IRI but perhaps by differing mechanisms.⁹

The complement system is very tightly regulated to prevent damage to self by circulating and membrane-bound regulatory proteins. The latter act at two levels within the complement cascade; at the levels of the C3 convertase enzyme and MAC. Decay accelerating factor (DAF, CD55), membrane co-factor protein (MCP, CD46), and, in addition, in rodents, Crry, act at the C3 and C5 convertase levels whereas CD59 is the only membrane-bound factor that prevents the formation of C5b-9.¹⁰ CD59 is an 18- to 20-kD glycosyl-phosphatidylinositol-anchored protein that is ubiquitously expressed.^{11,12} Its mechanism of action is to inhibit the interaction of C8 with C9, thereby preventing pore formation in cell membranes.¹⁰ In contrast to humans, mice have two CD59 genes encoded on chromosome 2; *mCd59a* and *mCd59b*. *mCd59b* predominates in the testes, whereas *mCd59a* is the major regulatory isoform in kidney, brain, and liver.^{13,14} Within the kidney CD59a is expressed within distal tubules, collecting ducts, vascular endothelium, and in glomeruli.¹³ Mice with a targeted deletion of the *mCd59b* gene have recently been reported to develop a severe hemolytic anemia and progressive male infertility whereas mice lacking the *mCd59a* gene (*mCd59a*−/−) developed a mild, spontaneous, intravascular hemolysis.^{15,16} We have recently found that *mCd59a*−/− mice are more susceptible to accelerated nephrotoxic nephritis, a model of immune complex-mediated nephritis, demonstrating the important function of CD59 in protecting the glomerulus from complement-mediated damage *in vivo*.¹⁷ However the role of CD59 in tubulointerstitial injury such as that secondary to ischemia has not been fully elucidated. In this study, we used *mCd59a*−/− mice and demonstrated that *mCd59a* deficiency exacerbated tubulointerstitial damage after renal IRI. In particular *mCd59a*−/− mice developed more severe tubular necrosis and apoptosis and greater leukocyte infiltration than genetically matched wild-type (WT) controls suggesting that CD59a plays an important protective role *in vivo*.

Materials and Methods

Animals

CD59a-deficient mice were generated as previously described.¹⁶ Male *mCd59a*−/− mice, backcrossed for six generations onto a C57BL/6 genetic background, were compared with sex- and age-matched C57BL/6 WT⁴ animals for each experiment. Mice were kept in a specific, pathogen-free environment. All animal procedures were performed in accordance with institutional guidelines.

Ischemia Protocol

Mice were anesthetized by inhalation of Isoflurane (Abbott Laboratories Ltd., Berkshire, UK). Additional analgesia was administered by preoperative intraperitoneal in-

jection of 0.2 mg of buprenorphine in 400 μ l of phosphate-buffered saline (Vetergesic; Alstoe Ltd., Melton Mowbray, UK) to prevent postoperative pain. Body temperature was kept constant by placing a warm pad beneath the animal. After midline abdominal incision, the left renal pedicle was bluntly dissected and a microvascular clamp (B1 clamp, 00396-01; Fine Science Tools, Heidelberg, Germany) was applied for 30 minutes. This period of ischemia was predetermined (for this anesthetic protocol) using a range of occlusion times of 30 to 60 minutes and found to give reversible injury. After occlusion, 0.8 ml of prewarmed saline was placed in the peritoneal cavity and the abdomen was closed. The left kidney was observed for an additional 1 minute after removal of the clamps to see the color change indicative of blood reflow. After suturing peritoneum and skin, the mice were returned to their cages with free access to food and water. Mice were sacrificed at 72 hours or 2 weeks and both kidneys were harvested for assessment of renal injury. The right (nonischemic) kidney was used as an internal control for each mouse.

Histological Analysis

Kidneys were fixed for 24 hours in buffered formal saline, transferred to 70% ethanol, and embedded in paraffin. Sections were stained with periodic acid-Schiff (PAS) or hematoxylin and eosin. All analyses were performed blinded to the sample identity. Tubulointerstitial damage was assessed by ranking of sections taking into account tubular necrosis, tubular dilatation, and cast formation in randomized sections. A score of tubular cast formation was calculated as follows: casts in 0 to 25% of tubules within each cortical or medullary high-powered field (HPF) = 1; 25 to 50% = 2; 50 to 75% = 3; 75 to 100% = 4. Fifteen HPFs⁴ were counted for each section of either cortex or medulla. Interstitial neutrophils were identified by typical nuclear morphology and quantified by counting the number of neutrophils in each of 20 HPFs per section.

Immunofluorescence

Kidneys were snap-frozen in isopentane cooled in liquid nitrogen and stored at −70°C. Frozen sections were cut at a thickness of 5 μ m. An observer without knowledge of the sample identity performed all quantitative immunofluorescence analyses. For C9 staining a rabbit anti-rat C9 primary antibody (1:100) (cross-reactive with mouse C9¹⁸) and a fluorescein isothiocyanate-conjugated mouse anti-rabbit IgG secondary (1:50) (Sigma, Gillingham, UK) were used. In quantitative immunofluorescence studies, to exclude artifacts because of variable decay of the fluorochrome all sections from one experiment were stained and analyzed at the same time. Sections were examined at $\times 400$ magnification using an Olympus BX4 fluorescence microscope (Olympus Optical, London, UK). A Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, UK) was attached to the microscope and, using Image-Pro Plus software (Media

Cybernetics, Silver Spring, MD), images were captured for analysis. For each section 15 HPFs were examined and the mean fluorescence intensity was recorded, with results expressed in arbitrary fluorescence units.⁴

Immunohistochemistry

For macrophage staining a primary monoclonal rat anti-mouse CD68 antibody (1:50) (Serotec, Oxford, UK) was used on frozen sections. Sections were blocked with a 1% solution of hydrogen peroxide in 50% methanol. A mouse anti-rat secondary (1:200) (Jackson Immuno-research, Cambridge, UK) and a rat peroxidase anti-peroxidase tertiary antibody (1:100) (Jackson Immuno-research, Cambridge, UK) were then applied. The sections were developed with diaminobenzidine (Sigma) and counterstained with hematoxylin (Sigma). Macrophage staining was assessed by quantifying mean intensity of staining per HPF again using Image-Pro Plus software. Lymphocytes were stained with a phycoerythrin-conjugated anti-mouse CD45RB antibody (1:100) (Pharmin-gen, Oxford, UK) and quantified by counting the number of lymphocytes per HPF averaged over 20 HPFs per section. Apoptosis was assessed by terminal dUTP nick-end labeling (TUNEL) staining using the *In Situ* Cell Death Detection kit, POD (Roche Applied Science, East Sussex, UK) following the manufacturer's instructions and quantified by counting the number of apoptotic nuclei per HPF averaged over 20 HPFs per section.

Statistical Analysis

All values described in the text and figures are expressed as median and range for *n* observations. Statistical analysis was performed using GraphPad Prism 3.02 (Graph-Pad Software, San Diego, CA). Data were analyzed using the Wilcoxon pairs test for all comparisons and a *P* value of less than 0.05 was considered to be significant.

Results

mCD59a^{-/-} Mice Were More Susceptible to IRI

We initially studied mice killed 72 hours after IRI to ascertain whether the lack of CD59 would influence the early tubular damage and neutrophil influx typical of this stage of the disease process. Renal sections from WT C57BL/6 mice displayed moderate tubular injury characterized by loss of the epithelial brush border, flattening of epithelial cells, necrotic tubular cells, and occasional tubular casts predominantly in the corticomedullary region (Figure 1, A and C). However, mCD59a^{-/-} developed significantly more severe tubular damage (median rank of tubular injury, 10; range, 6 to 14; *n* = 8 for mCD59a^{-/-} versus 4; 1 to 11; *n* = 8 for WT, *P* = 0.01; Figure 1, B and D, and Figure 2A) that at times extended into the cortex with a significantly greater number of

Figure 1. Representative light microscopy of PAS-stained renal sections after renal IRI. The WT mouse kidneys (A and C, 72 hours after IRI; E and G, 2 weeks) showed a modest cellular infiltrate with minor tubular injury limited to the corticomedullary area. In contrast, mCd59a^{-/-} mouse kidneys (B and D, 72 hours; F and H, 2 weeks) displayed an impressive, predominantly neutrophil cellular infiltrate and severe tubular damage and cast formation. Original magnifications: $\times 100$ (A, B, E, F); $\times 400$ (C, D, G, H).

tubular casts in the medulla (median cast score, 3.0 per HPF; range, 2.0 to 3.0; *n* = 8 for mCd59a^{-/-} versus 2.0; 1.0 to 3.0; *n* = 8 for WT, *P* < 0.05; Figure 2B). There were few cortical tubular casts in either group. The excess tubular injury in the mCd59a^{-/-} mice was accompanied by considerably greater interstitial polymorphonuclear cell infiltrate when compared with the WT controls (median neutrophil count, 12.2 per HPF; range, 6.3 to 33.2 for mCd59a^{-/-} versus 3.5; 0.8 to 8.7 for WT, *P* = 0.04; Figure 2C). Importantly, the right (nonischemic) kidney of each mouse did not display any morphological abnormalities on light microscopy suggesting that neither the general anesthetic nor surgical complications were likely to play a major role in the pathogenesis of the histological changes (data not shown).

Figure 2. Tubular injury rank, tubular cast formation, and neutrophil influx in mice 72 hours after renal IRI. **A:** Tubular injury score was significantly greater in *mCd59a*^{-/-} animals than in matched controls. **B:** Quantitative analysis of PAS-stained sections revealed that tubular cast formation was greater in *mCd59a*^{-/-} mice than in WT animals in the medulla but not the cortex of the kidney. **C:** A marked increase in neutrophils was also seen in *mCd59a*^{-/-} animals compared with controls. The error bars denote SEM.

mCd59a^{-/-} Mice Developed More Apoptosis

Because MAC is capable of inducing apoptosis in several models of experimental nephritis,¹⁹ we examined whether the absence of CD59a, which we would expect to allow increased MAC synthesis, would worsen apoptosis. Apoptotic cell nuclei were clearly seen in PAS-stained sections of both WT and *mCd59a*^{-/-} mice, pre-

Figure 3. TUNEL staining of apoptotic cells within the tubulointerstitium of WT (**B**) and *mCd59a*^{-/-} (**C**) mice. **A:** Significantly more TUNEL-positive cells were seen in the tubulointerstitium of *mCd59a*^{-/-} animals compared with matched controls at 72 hours after IRI. Error bars denote SEM.

dominantly concentrated in areas of most severe renal damage. To assess the degree of apoptosis in more detail we performed TUNEL staining, which demonstrated more apoptotic cells in the *mCd59a*^{-/-} mice than matched controls (median number of apoptotic cells per HPF, 8.8; range, 7.2 to 26.9 for *mCd59a*^{-/-} versus 3.0; 2.2 to 10.1 for WT, *P* = 0.02; Figure 3).

CD59 Protects from Renal MAC Deposition in Vivo

To further analyze the potential mechanism whereby lack of CD59 exacerbated renal injury we studied the expression of the terminal complement component C9 as a marker of MAC deposition. *mCd59a*^{-/-} mice deposited significantly more C9 (32.1 arbitrary fluorescence units; range, 25.3 to 40.5 for *mCd59a*^{-/-} versus 19.3; 13.8 to 20.9 for WT, $P = 0.03$; Figure 4) within the tubulointerstitium than controls. Deposition of C9 occurred predominantly in a peritubular manner in all sections (Figure 4).

mCd59a^{-/-} Mice Did Not Recover from Initial IRI

IRI is characterized by an initial neutrophil infiltrate and tubular damage followed by mononuclear cell infiltration, tubular cell regeneration, and eventual recovery. In view of our initial findings of excess tubulointerstitial damage in *mCd59a*^{-/-} animals we decided to investigate whether *mCd59a* deficiency would also exacerbate the subsequent recovery phase of the disease. We therefore studied mice at 2 weeks after the ischemic insult. Tubular injury at this time point was significantly worse in *mCd59a*^{-/-} mice than controls (median rank of tubular injury, 5.5; range, 2 to 9; $n = 8$ for *mCd59a*^{-/-} versus 2.5; 1 to 8; $n = 8$ for WT, $P = 0.02$) (Figure 1, F and H, and Figure 5A). Indeed in a proportion of the WT animals almost no renal damage was seen morphologically (Figure 1, E and G). In keeping with the excess tubular damage, interstitial lymphocyte infiltration was significantly greater in the *mCd59a*^{-/-} mice than WT animals (median lymphocyte count, 21.3 per HPF; range, 15.1 to 39.9 for *mCd59a*^{-/-} versus 11.5; 9.4 to 13.2 for WT, $P = 0.02$; Figure 5B and Figure 6). In contrast, there was no difference in macrophage counts between the groups of mice (median intensity per HPF, 0.082; range, 0.02 to 0.15 for *mCd59a*^{-/-} versus 0.019; 0.00 to 0.22 for WT, ns; Figure 5C). To corroborate the data at 72 hours that CD59 protects against MAC formation *in vivo* we again stained renal sections for C9. This confirmed the findings at 72 hours after IRI that *mCd59a*^{-/-} mice deposit significantly more C9 (35.8 arbitrary fluorescence units; range, 26.5 to 51.1 for *mCd59a*^{-/-} versus 20.2; 13.8 to 23.8 for WT, $P = 0.001$; Figure 5D) within the tubulointerstitium than controls again occurring in a peritubular manner in all sections.

Discussion

In this report we have demonstrated that mice lacking the *mCd59a* gene were more susceptible to IRI than matched controls. Specifically, they displayed greater tubular damage and apoptosis and more neutrophil influx early in the disease process. Additionally, considerable renal injury persisted in *mCd59a*^{-/-} mice at a later time point when compared to controls in terms of both tubular injury and lymphocyte infiltration. The excess C9 deposition

Figure 4. Immunofluorescence for C9 as a marker of MAC. C9 staining occurred predominantly in a peritubular distribution in both WT and *mCd59a*^{-/-} mice. Quantitative analysis of these immunofluorescent sections revealed significantly more C9 deposition in *mCd59a*^{-/-} mice than controls at 72 hours after IRI. Error bars denote SEM.

that we observed reflects the presence of a greater quantity of MAC and most likely represents the mechanism whereby the absence of CD59a caused greater tissue injury.

There are several lines of evidence that implicate complement activation in the etiopathogenesis of IRI. Firstly, deposition of complement C3 fragments and MAC consistently occur within hours of IRI in any organ affect-

Figure 5. Tubular injury score, lymphocyte and macrophage counts, and C9 staining within the kidneys of mice 2 weeks after renal IRI. **A:** Tubular injury was significantly greater in *mCd59a*^{-/-} animals than WT controls. **B:** Quantitative analysis of immunofluorescent sections revealed considerably more lymphocytes per HPF in CD59-deficient than in WT mice. **C:** There was, however, no difference in macrophage number between these groups of mice. **D:** Additionally, a marked increase in C9 staining was seen in *mCd59a*^{-/-} animals compared with matched controls. Error bars denote SEM.

ed.^{20,21} Secondly, general complement consumption such as using cobra venom factor reduces injury in for example, cardiac IRI.^{21,22} Additionally, in most organs studied, there seems to be a role for both C5a and MAC in causing tissue injury after a reversible ischemic insult. The evidence for this role has been elucidated from the use of animals deficient in either C5 or C6 or by the utilization of inhibitors of C5.^{23–27} The relative importance of MAC compared with C5a in causing tissue damage in renal IRI depends to a large extent on the protocol and species used when inducing the initial injury. A recently performed series of experiments using mice deficient in complement components C4, C3, C5, and C6 showed that complement activation, presumably via the alternative pathway, mediates, at least in part, tubular injury after renal IRI. The same study demonstrated that the predom-

inant effect on both tubular damage and neutrophil accumulation occurred via MAC rather than C5a.⁷ Other groups however, have not found such clear-cut differences, additionally highlighting an important role for C5a in causing a cellular infiltrate and some of the tubular damage (but not apoptosis) in IRI.^{9,28} Our data add to the weight of evidence that MAC is importantly involved in the pathogenesis of both the cellular influx and tubular cell death via apoptosis and necrosis after renal IRI in mice.

The complement cascade is tightly controlled predominantly at the levels of the C3 convertases and MAC. The role of these complement regulatory proteins in tubulointerstitial damage has not been extensively investigated. One recent study, using a Crry-Ig fusion protein found no benefit of infusion of Crry-Ig in preventing renal injury despite the fact that C3-deficient animals were protected from IRI.²⁹ Possible explanations for this lack of effect may be that Crry-Ig remained predominantly within the vascular endothelium and did not reach the tubular compartment where most of the damage in renal IRI seems to occur. Additionally, in contrast to most other studies of renal IRI in mice, the investigators clamped the renal arteries alone, leaving the renal veins patent. This could potentially alter the hemodynamic effects of the ischemic insult, which in turn may alter the relative importance of complement activation and/or complement regulators in the pathogenesis of the subsequent tissue damage. In contrast to the above report the results presented here clearly emphasize the role that the complement regulator CD59a, plays in protecting the tubules from ischemic injury in mice.

In addition to lysis, complement-mediated damage by MAC can occur by the insertion of sublethal quantities of C5b-9 into cell membranes. It has been demonstrated that sublethal MAC can mediate proliferative, proinflammatory, profibrotic, and proapoptotic effects *in vitro* in renal cells.^{30–42} MAC has been shown to induce the synthesis and/or secretion of a number of proinflammatory cytokines such as tumor necrosis factor- α and interleukin-1,^{41,43} adhesion molecules such as ICAM-1 and E-selectin,⁴² and chemokines, eg, interleukin-8.^{6,44} These effects, particularly leukocyte chemotaxis secondary to interleukin-8, may play a role in the excess infiltration of both neutrophils and lymphocytes into the kidney seen in CD59a-deficient animals in our study. In all these experiments surgery was performed only on the left kidney allowing the right kidney to be used as an internal control. Therefore we would not have expected to detect differences in renal functional parameters.

In contrast to the effects of C5a, MAC is able to induce apoptosis in tubular cells *in vitro* and *in vivo*.^{19,45,46} Inhibition of C5a by using a specific C5a receptor antagonist had no influence on renal apoptosis in a murine model of IRI.⁹ We have demonstrated that the excess MAC deposition in our *Cd59a*^{-/-} mice after IRI was associated with significantly more renal apoptosis than matched controls suggesting that MAC plays a predominant role in inducing tubular cell apoptosis.

After the submission of this article, a report appeared by Yamada and colleagues⁴⁷ that examined the effects of deficiency of DAF, CD59a, or both after IRI. In agree-

Figure 6. Representative immunofluorescence staining for lymphocytes 2 weeks after renal IRI. The mononuclear cell infiltrate seen in PAS-stained sections was shown to be predominantly lymphocytes when stained for using an anti-CD45RB antibody and was greater in *mCd59a*^{-/-} mice (**B**) than controls (**A**).

ment with the findings described in our study, they observed greater renal injury in mice deficient in both CD59a and DAF compared to mice deficient in DAF alone suggesting an important role for CD59a in protecting the kidney from complement-mediated damage. However, in contrast to our study, they found no differences in the degree of kidney damage between mice deficient in CD59a and controls. This apparent discrepancy may be explained by differences in methodology between the two studies such as route of anesthetic administration, surgical technique, or length of ischemic insult. More importantly they studied mice 24 hours after ischemic injury whereas we examined renal tissue at 72 hours and 2 weeks after the initial insult. This enabled us to investigate the function of the terminal complement pathway in the later recovery phase of IRI. We additionally demonstrated that CD59a plays an important role in preventing cellular apoptosis and mononuclear cell infiltration into the tubulointerstitium in this clinically relevant model of renal injury.

In summary, we have demonstrated that CD59a, by preventing the deposition of MAC, reduces the severity of renal damage after IRI in mice.

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