Complement Factor C5a Mediates Renal Ischemia-Reperfusion Injury Independent from Neutrophils


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The complement system has been shown to mediate renal ischemia-reperfusion (I/R) injury. However, the contribution of complement factor C5a to I/R injury, in particular in the kidney, remains to be established. In this study, we investigated the impact of blocking the C5aR pathway on the inflammatory response and on the renal function in a murine model of I/R injury. First, we analyzed C5aR expression in kidneys of healthy mice. Intriguingly, we found expression on mesangial, as well as on tubular epithelial, cells. After I/R injury, C5aR expression was up-regulated in tubular epithelial cells. In addition, mRNA levels of CXC chemokines and TNF-α increased significantly and kidneys were heavily infiltrated by neutrophils. Blocking the C5aR pathway by a specific C5a receptor antagonist (C5aRA) abrogated up-regulation of CXC chemokines but not of TNF-α and reduced neutrophil infiltration by >50%. Moreover, application of the C5aRA significantly reduced loss of renal function. This improvement of function was independent of the presence of neutrophils because neutrophil depletion by mAb NIMP-R14 did not affect the protective effect of C5aRA treatment. Furthermore, blocking of the C5aR pathway had no influence on renal apoptosis. These data provide evidence that C5a is crucially involved in the pathogenesis of renal I/R injury by modulation of neutrophil-dependent as well as neutrophil-independent pathways, which include the regulation of CXC chemokines but not TNF-α or apoptotic pathways. The Journal of Immunology, 2003, 170: 3883–3889.

Organ injury as a consequence of ischemia followed by reperfusion is a major clinical problem. Renal ischemia-reperfusion (I/R) injury is the most common cause of acute renal failure as seen after renal transplantation, major abdominal and vascular surgery, coronary bypass surgery, and in trauma and sepsis (1). I/R injury is characterized by the massive influx of neutrophils, which are believed to play a crucial role in the pathophysiology of posts ischemic renal failure (2, 3). The infiltration of neutrophils into tissue sites of inflammation is regulated by CXC chemokines such as IL-8, Groα, of which cytokine-induced neutrophil chemoattractant (KC) is the murine homolog, and macrophage inflammatory protein-2 (MIP-2) (4).

The complement system has also been shown to be an important mediator of I/R injury (5–7). Both the formation of membrane attack complex (MAC) as well as the generation of the anaphylatoxin C5a can potentially mediate I/R injury. The MAC has been reported to mediate neutrophil influx, synthesis of proinflammatory cytokines and may cause direct cell injury, apoptosis, and necrosis (8–10). Responses to C5a are mediated by the C5aR, a member of the rhodopsin family of seven transmembrane-spanning G-protein-linked receptors (11). Activation of C5aR induces recruitment of neutrophils and macrophages, and activates these and other cells to produce cytokines, chemokines, and adhesion molecules (12, 13). Recent work has also attributed a role to C5a in the regulation of apoptosis (14, 15).

Recently, we showed that inhibition of the complement system by inhibition of C5 activation, preventing C5a generation as well as MAC formation, strongly protects against renal I/R injury (16). Inhibition of C5 abrogated the induction of chemokines and influx of neutrophils and abolished I/R-induced late apoptosis. Renal I/R injury has in particular been attributed to the formation of MAC, whereas C5a has been demonstrated to be involved in I/R injury of the heart and the intestine (5, 17–20). No specific data currently exist concerning the role of C5a in renal I/R.

Therefore, we analyzed the role of C5a in I/R injury using a specific C5aR antagonist. Our findings give evidence that the C5aR pathway becomes activated in I/R injury and mediates renal dysfunction as a consequence of a strong C5a induced inflammatory response and tissue damage, which involves neutrophil-dependent, as well as neutrophil-independent, pathways.

Materials and Methods

Abs and reagents

For in vivo studies, a C5a receptor antagonist (C5aRA) was used. This C5aRA has been shown to neutralize C5a function in vitro and in vivo (20). NIMP-R14 (rat anti-mouse neutrophil mAb) was kindly provided by Dr. M. Strath (National Institute for Medical Research, London, U.K.). This mAb has an in vivo neutrophil-depleting capacity (21). Rabbit anti-mouse C5aR polyclonal Ab (C5aRA) was a kind gift from Dr. J. van Beek (College of Medicine, University of Wales, Cardiff, U.K.). Secondary peroxidase-conjugated goat anti-rabbit and goat anti-rat IgG Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated and Texas Red-conjugated secondary Abs were purchased from...
PickCell Laboratories (Amsterdam, The Netherlands) and Southern Bio-technology Associates (ITK Diagnostics, Uithoorn, The Netherlands), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental protocol

Male Swiss mice weighing 20–25 g were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Animals were housed individually in standard laboratory cages and were allowed free access to food and water throughout the experiments. The studies were conducted under a protocol approved by the Institutional Animal Care Committee of the University of Maastricht (Maastricht, The Netherlands). At the start of the experiments, mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.). Body temperature was maintained at 39°C by a heating pad until animals recovered from anesthesia. Under aseptic conditions a 1.0-cm long midline abdominal incision was made and ischemia was induced by applying a nontraumatic vascular clamp to the left renal pedicle for 45 min. Subsequently, the wound was covered with cotton soaked in sterile PBS. After removal of the clamp, the left kidney was inspected for restoration of blood flow and the contralateral kidney was removed. The wound was closed in two layers and 0.25% bupivacaine was applied topically for postoperative pain management. The animals were sacrificed at indicated time points after reperfusion. At the time of sacrifice, blood was collected and the left kidney was harvested for analysis. Renal tissue was divided into representative pieces for the different assays. For immunohistochemistry, total cross-sections were embedded in OCT compound and immediately frozen in isopentane/dry ice. For apoptosis and mRNA assays, kidney pieces were snap-frozen in liquid nitrogen. Tissue samples were divided in four equal parts in a standardized fashion resulting in similar cortex-medulla ratios in each sample.

Mice (n = 6 per group) were subjected to ischemia and treated with 5°C for 1.5 h. Pharmacological interventions were given i.p., based on pilot experiments, 15 min before removal of the clamps, 1 and 2 h after reperfusion (3 × 100 μL of 1.8 × 10^8 M C5aRα dissolved in PBS, or 3 × 100 μL of PBS). In separate experiments, neutrophils were depleted using mAb NIMP-R14 (1 mL of 1 mg/ml in PBS), i.p. 6 h before induction of ischemia, which has been shown to deplete neutrophils for several days (21).

Renal histology

Cryostat sections (5 μm) of frozen tissue were fixed with acetone and stained for C5aR, using a rabbit anti-mouse C5aR polyclonal Ab, and for neutrophils, using mAb NIMP-R14. Staining was either visualized by 3-amino-9-ethylcarbazole followed by hematoxylin counterstain or immunofluorescence. No significant staining was detected in slides incubated with control rabbit serum (for C5aR) and rat IgG (for NIMP-R14) instead of the respective Ab. Quantitative analysis was performed by comparison of band intensities of the PCR products with standard curves prepared by PCR amplifications on dilution series of a highly concentrated murine renal cDNA.

Real-time quantitative RT-PCR for TNF-α was performed on a Taqman ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). β-actin was used as reference gene. The following oligonucleotide primers and probes were used: β-actin, 5′-GAC AGG ATG CAG AAG GCT-3′ (antisense) and 5′-CGC TCG CTT TGT GCA-3′ (sense) and 5′-ATT TTC AGG ATG CAG AAG GATT ACT ATT G-3′ (antisense) and 5′-CCA CCG ATC CAC ACA GAG TAC TT-3′ (antisense) both at a concentration of 300 nM, and internal fluorescence-labeled probe (JOE) 5′-ATC AAT AAG ATT ATT CCT CCT CGT GAG CGC-3′ at a concentration of 200 nM, and internal fluorescence-labeled probe (FAM) 5′-CAC GTG GTA GCA AAC CAA GTG GA-3′ at a concentration of 100 nM. All primers and probes were obtained from Applied Biosystems.

Results

The C5aR is widely expressed in the kidney

First, the renal C5aR mRNA expression was examined by RT-PCR. In kidneys of healthy male Swiss mice we found clear expression of C5aR. After renal I/R, C5aR mRNA levels increased evidently (Fig. 1A). Immunohistochemical staining for C5aR in healthy kidney revealed a faint expression on tubular epithelial cells and more intense staining of mesangial cells (Fig. 1B). After a 24-h reperfusion, C5aR expression in ischemic kidneys was up-regulated on tubular cells. In addition, infiltrating neutrophils stained positive for the C5aR (Fig. 1C).

To verify the hypothesis that during renal I/R tubular epithelial cells, in addition to infiltrating inflammatory cells, express the C5aR, we performed a double-staining using immunofluorescence on tissue sections of kidneys subjected to ischemia followed by a 24-h reperfusion. Fig. 2A shows that renal tissue is infiltrated by neutrophils. The tubular epithelial cells show a strong positive staining for the C5aR (Fig. 2B). The overlay picture (Fig. 2C) shows that not only do all infiltrating neutrophils express the C5aR but that tubular epithelial cells also express C5aR.
Blocking the C5aR pathway attenuates I/R-induced renal dysfunction

Next, the functional role of C5a in the pathophysiology of renal I/R injury was studied. BUN and serum creatinine, as a measure for renal dysfunction, increased significantly from median values of 9.5 mmol/L (BUN) and 80.4 µmol/L (creatinine) in healthy controls to 58.3 mmol/L (BUN) and 245.3 µmol/L (creatinine) in animals subjected to renal ischemia followed by 24 h reperfusion. Treatment with the C5aRA significantly reduced median BUN concentrations from 58.3 to 27.3 mmol/L as well as serum creatinine values from 245.3 to 150.2 µmol/L (p < 0.05) 24 h after I/R injury (Fig. 3, A and B, respectively).

Neutrophil-influx in renal I/R injury depends mainly on the C5aR pathway

A key feature of I/R injury is the influx of neutrophils. In fact, renal I/R induced a significant influx of neutrophils (median of 5.1 polymorphonuclear cells (PMNs) per field of vision) 24 h after reperfusion. Treatment with the C5aRA significantly reduced the influx of neutrophils, to a median value of 2.3 PMNs per field of vision, compared with control-treated animals (p < 0.05; Fig. 4). Infiltrating neutrophils mainly localized to the corticomedullary region after renal I/R, the inhibitory effect of C5aRA was most pronounced in these regions (data not shown). To further analyze the mechanism of C5a-mediated I/R injury, the role of neutrophils in the course of renal I/R injury was investigated. For this purpose, neutrophils were depleted before the induction of ischemia by administration of mAb NIMP-R14, 6 h before renal ischemia. Neutrophil-depleted mice received either the C5aRA or control (PBS) treatment. To our surprise, neutrophil depletion did not protect against I/R-induced renal dysfunction (Fig. 3). Renal function did not differ between neutrophil-depleted animals and control-treated animals (median BUN values of 56.4 vs 58.3 mmol/L and serum creatinine 245.3 vs 247.5 µmol/L; both p > 0.5). These data suggest that neutrophils are not essential for renal dysfunction in the course of renal I/R injury. However, application of the C5aRA significantly protected neutrophil-depleted mice from renal failure.

BUN and serum creatinine values decreased significantly from 56.4 (BUN) and 245.3 (serum creatinine) in PMN-depleted mice to 24.7 mmol/L and 125.2 µmol/L in C5aRA-treated mice (p < 0.05; Fig. 3, A and B, respectively). These data indicate that infiltration of neutrophils alone is not sufficient to mediate renal I/R injury. Moreover, our results give evidence that activation of the complement system and, in particular, triggering of the C5aR pathway is a crucial mechanism inducing renal dysfunction in I/R injury.

Induction of the CXC chemokines KC and MIP-2 and TNF-α in the course of renal I/R

C5a is a powerful chemoattractant and can induce neutrophil influx. C5a is also known to be involved in the induction of chemokine and cytokine production. In this context, we investigated the renal expression of the murine chemokines KC and MIP-2 in the absence or the presence of the C5aRA by semiquantitative PCR techniques. Renal I/R induces a 2- and 5-fold up-regulation of renal mRNA levels of the respective murine CXC chemokines KC and MIP-2 at a 24-h reperfusion as compared with healthy controls (Figs. 5, A and B, respectively). Treatment with the C5aRA completely prevented the up-regulation of KC and MIP-2 mRNA (Fig. 5, A and B, respectively). These data give evidence that C5a plays an important role in the regulation of chemokine induction in the course of renal I/R injury.
Another important mediator of renal I/R injury is TNF-α. TNF-α mediates the induction of chemokines and subsequent neutrophil influx, but is also involved in the induction of apoptosis. Therefore, we assessed the renal expression of TNF-α upon renal I/R by real-time and semiquantitative PCR. Data did not differ between real-time and semiquantitative PCR, thus only real-time PCR data are shown. Renal I/R induced a significant 2.5-fold increase of TNF-α mRNA \( (p < 0.05) \). Interestingly, treatment with C5aRA did not affect this local up-regulation of TNF-α in the course of renal I/R injury (Fig. 5C) suggesting that the C5a-mediated up-regulation of KC and MIP-2 mRNA is independent of TNF-α.

C5aRA does not effect I/R-induced apoptosis

Renal apoptosis is an important feature in renal I/R injury. In this study, we show that renal apoptosis occurred as early as 2 h after reperfusion which was still ongoing at 24 h (Fig. 6). Treatment with the C5aRA has no influence on the induction of renal apoptosis, neither after 2 h nor after 24 h of reperfusion. Thus, C5a appears to be not involved in renal apoptosis in the course of renal I/R injury.

Discussion

The complement system has been implicated in several models of I/R injury (5–7). In myocardial I/R models, recent work indicates that the mannose-binding lectin, as well as the classical activation pathway, plays a role in complement activation and subsequent organ damage (22, 23). Regarding the precise pathway of complement activation upon renal I/R injury much remains to be resolved, however evidence is growing that activation of the complement system plays a crucial role in the pathogenesis of renal I/R injury (5). Recently, we showed that inhibition of the activation of complement factor C5 is strongly protective against the development of tissue injury upon renal I/R, indicating that the complement system indeed plays a crucial role in the development of renal I/R injury (16). To further unravel the role of the complement system, the involvement of C5a and its receptor in the pathophysiology of renal I/R injury was investigated. This study shows that C5aRA significantly attenuates renal I/R injury. This appears to be in contrast to studies by Zhou et al. (5) who provided evidence for a crucial involvement of the MAC in renal I/R injury using transgenic mice. They showed that C3, C5, but also C6-deﬁcient mice are protected against tissue damage upon renal I/R. Moreover, inhibition of C5, preventing C5a generation, in C6-deﬁcient mice did not have additional protective effects. On the basis of these data, Zhou et al. (5) concluded that renal I/R injury is solely dependent on formation of MAC. However, the lack of additional effects of anti-C5 in these C6-deﬁcient mice could well be explained by codominant effects of C5a and MAC as previously reported for C5a and IgG FcγRs (24). Furthermore, the model used by Zhou et al. (5) cannot be directly compared with the one used in this study because the experimental setting was different. These authors per-
formed bilateral warm ischemia with a duration of up to 1 h, in complement-deficient mice with different backgrounds, among others C57BL/6 mice, a mouse strain known to be sensitive for renal I/R injury (5, 25). Recently, Park et al. (26) showed that complement inhibition, using the rodent C3-convertase inhibitor Crry-Ig, does not protect against renal I/R in the mouse, suggesting that the complement system is not essentially involved in renal I/R injury. The model used in this elegant study is characterized by a very short duration of renal ischemia (20–30 min) and heparin pretreatment. In contrast, studies of Zhou et al. (5) and ours, using longer ischemia times, showed that the complement system is crucially involved in renal I/R injury. Taken together, these studies indicate that the experimental models used, and especially ischemia times, play an important role in the involvement of the complement system and the effect of complement inhibition on I/R injury. This is in line with recent work of Iwata et al. (27) who showed that duration of ischemia crucially determines the mechanisms involved in I/R injury. Whereas I/R injury due to short ischemic insults is mainly mediated by neutrophils, after longer ischemic insults, apoptosis plays a crucial role in the development of I/R injury. Previously, we also showed the important pathogenic role of apoptosis in our 45-min renal I/R model (28). These data suggest that the complement system is most crucially involved in I/R injury due to more severe, clinically relevant, ischemic insults.

In this study, we indeed show that the application of a specific C5aRA strongly reduces renal I/R injury implicating an important pathophysiological role of this complement cleavage product.

C5a functions via the specific C5aR, a member of the rhodopsin family of seven transmembrane-spanning G-protein-linked receptors (11). The C5aR was originally shown to be present on myeloid cells, among others neutrophils and macrophages (11). Binding of C5a to the C5aR induces chemoattraction and degranulation of leukocytes. It also functions as an anaphylatoxin, inducing smooth muscle cell contraction, histamine release from mast cells, vasodilatation, and increased vascular permeability. More recent work has shown that C5aR is also present on nonmyeloid cells, in particular bronchial and alveolar epithelial cells, hepatocytes, endothelial cells, and also in renal tissue on mesangial and (proximal) tubular epithelial cells (30). In this study, we report the presence of the C5aR on mesangial cells and tubular epithelium of the murine

**FIGURE 5.** The C5aRA inhibits I/R-induced up-regulation of KC and MIP-2 mRNA levels (A and B, respectively) as measured by semiquantitative PCR. Renal I/R induces an evident up-regulation of KC and MIP-2 at 24 h reperfusion (A and B, respectively). Treatment with C5aRA abrogates the up-regulation of KC and MIP-2 mRNA. Data shown are medians with interquartile ranges (n = 4 per group) calibrated against equal amounts of β-actin mRNA. For TNF-α mRNA levels, real-time PCR was used (C). Renal I/R induces a significant up-regulation of TNF-α which is unaffected by C5aRA treatment (C). Data shown are medians with interquartile ranges (n = 6 per group) calibrated against β-actin mRNA levels. Statistical significance as compared with healthy controls was denoted at p < 0.05 (*).

**FIGURE 6.** The C5aRA does not effect I/R-induced apoptosis. The extent of renal apoptosis is reflected by fragmented DNA amplified by LM-PCR and visualized on ethidium bromide-stained gel. In PBS-treated animals, internucleosomal DNA cleavage was evident after 2 and 24 h of reperfusion. Treatment with C5aRA did not effect internucleosomal DNA cleavage. Data shown are representative for three independent assays on different renal samples (n = 3 per group). On the left, a 100-bp m.w. marker is shown.
kidney, on mRNA as well as on the protein level. Moreover, we show that C5aR is locally up-regulated in the course of renal I/R injury. These findings are in line with the reported expression of C5aR in the human kidney (31, 32).

Administration of C5aRA strongly reduced the influx of neutrophils in the course of renal I/R injury, which is in line with the effects of C5aRA in other inflammatory models in vivo (17, 19, 20, 33). Neutrophils are considered to be crucially involved in the pathophysiology of I/R injury, nevertheless, the role of neutrophils in renal I/R injury remains controversial (2, 34). Amsterdam et al. (18) previously showed that inhibition of C5a, using a mAb against C5a, reduced infarct size in the course of cardiac I/R, without reduction of the influx of neutrophils, indicating that some of the effects of C5a inhibition are independent of infiltrating neutrophils (17, 29). In our study, neutrophil depletion did not effect the course of renal I/R injury, as was previously reported (35, 36) but rebuffed by others (3). Interestingly, the protective effects of C5aRA were independent of the influx of neutrophils, as neutrophil depletion in addition to C5aRA did not influence its protective effects. These data suggest that C5a has other receptor-mediated functions in the course of renal I/R injury, potentially mediated by C5aR expressed on mononuclear cells such as tubular epithelial cells, leading to renal function loss.

The induction of chemokines KC and MIP-2 in the course of I/R injury has been described previously and is reported to play a crucial role in neutrophil recruitment and subsequent I/R injury (3, 37, 38). In this study, we show for the first time that C5a is crucially involved in the regulation of these chemokines in the course of I/R. The importance of C5a in the induction of chemokines has been reported in a rat lung injury model induced by IgG immune complex deposition, however in this model, C5a-mediated chemokine induction depends on the presence of a costimulus (IgG immune complex) (12, 39). Interestingly, in these studies, C5aR did not mediate local TNF-α production (39). This is in line with our data showing that C5a is not involved in TNF-α production in the course of renal I/R injury.

Activation of the complement system has been implicated in the induction of apoptosis, a process which has been shown to be crucially involved in the pathophysiology of I/R injury (28). In particular, the MAC is regarded to induce apoptosis in several cellular models, including renal epithelial cells (12, 13). The MAC is regarded to induce apoptosis in several cellular models, including renal epithelial cells.

In conclusion, the data presented in this study demonstrate a crucial role for C5a in the pathophysiology of renal I/R injury. C5aRA significantly attenuates I/R-induced renal failure; C5aRA strongly reduces the influx of neutrophils and induction of chemokines. Interestingly, the protective effects of C5aRA are not dependent on the presence of neutrophils but rather on direct abrogation of renal functional loss possibly mediated by direct activation of renal epithelial cells.

References


