Role of CXC Chemokine Receptor 3 Pathway in Renal Ischemic Injury

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Chemokines play a major role in the recruitment of leukocytes in inflammation and in the regulation of T helper 1 (Th1)/Th2 immune responses. These mechanisms have been recognized to be important in the pathogenesis of renal ischemia-reperfusion (I/R) injury. The interaction of the CXC chemokine receptor 3 (CXCR3) receptor with its ligands is a key pathogenic pathway in promoting inflammation and in enhancing Th1 immune responses. After the induction of ischemia in the mouse model of renal ischemia, an increase in intrarenal expression of CXCR3 and its ligands was observed. Compared with the wild-type (WT) mice, CXCR3-deficient mice (CXCR3<sup>−/−</sup>) had significantly lower serum creatinine levels, better survival rate, and significantly less acute tubular necrosis and cellular infiltrates. In the kidney, intracellular staining of infiltrating cells that were recovered from kidneys revealed a lower percentage of CD4<sup>+</sup>IFN-γ<sup>+</sup> cells in the CXCR3<sup>−/−</sup> mice compared with the WT mice. Furthermore, adoptive transfer of WT CD3<sup>+</sup> cells into CXCR3<sup>−/−</sup> mice before induction of I/R injury abrogated the protection of CXCR3<sup>−/−</sup> mice from I/R injury. It is concluded that CXCR3 plays an important role in orchestrating the recruitment of Th1 cells to the ischemic kidney and in mediating I/R injury and therefore may serve as a novel target for the therapy of I/R injury.

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ischemia-reperfusion (I/R) injury could lead to ischemic renal failure, which is one of the most common forms of acute renal failure of the native kidneys as well as of the renal allograft (1–5). Despite major advances in understanding the pathogenesis of I/R, no satisfactory therapy is available, and ischemic acute renal failure remains a major cause of morbidity and mortality (6). I/R injury begins with tissue hypoperfusion/hypoxia and leads to depletion of cellular ATP and cytoskeletal damage (7,8). Restoration of blood flow is accompanied by reperfusion injury, which is characterized by production of a number of inflammatory molecules such as cytokines, reactive oxygen species (ROS), activation of leukocyte and endothelial cells, and upregulation of adhesion molecules/chemokines. The interactions of activated leukocytes with injured endothelial cells play a crucial role in the pathogenesis of the disease (6,9,10). There is a growing body of evidence indicating that ischemic renal failure is an inflammatory disease (6,11). Antineutrophil strategies, T cell depletion, and blockade of T cell CD28-B7 co-stimulation have been reported to be beneficial in protection against I/R injury (12–15). Chemokines are 8- to 10-kd proteins that play a central role in inflammation. They regulate all of the steps that are necessary for the recruitment of leukocytes to the sites of inflammation, including leukocyte activation, adhesion, chemotraction, and transmigration across the endothelial barrier (16–18). Chemokines are also important regulators of T helper 1 (Th1)/Th2 responses as Th1 chemokines selectively recruit Th1 cells to sites of inflammation (19–23). A Th1 shift has been shown to have a deleterious effect in the pathogenesis of I/R (24). The CXC chemokine receptor 3 (CXCR3) is expressed predominantly on a majority of activated inflammatory Th1 cells. It binds three different ligands, CXCL9 (monokine induced by IFN-γ [MIG]), CXCL10 (IFN-γ–induced protein-10 [IP-10]), and CXCL11 (IFN-γ inducible T cell α chemoattractant [ITAC]) (16). A high expression of CXCR3 receptor and its ligands has been reported in different models of inflammation, including rheumatoid arthritis, multiple sclerosis lesions, type 1 diabetes, and allograft rejection (25–28). Anti-CXCR3 strategies to reduce inflammation have yielded promising results in these disease models (24,25,29–33). The role of CXCR3 in the pathogenesis of I/R injury remains to be elucidated. Therefore, we tested the hypothesis that CXCR3 signaling plays a role in the induction of inflammation through Th1 cell infiltration, mediating renal I/R injury.

Materials and Methods

Animals

CXCR3<sup>−/−</sup> mice were generated in Craig Gerald’s laboratory (30). C57BL/6 mice, purchased from Jackson Laboratory (Bar Harbor, ME),
served as wild-type (WT) controls. All mice were weight (approximately 20 g) and sex matched in both groups. All procedures used in the animal experiments complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at Harvard University.

Uninephrectomized Renal I/R Injury Model

Mice were anesthetized by injection of 0.01 ml/g tribromoethanol, and a right flank incision was made. The right renal pedicle was divided between two ligatures, the right kidney was removed, and the wound was closed with staples. The mice were allowed to recover from anesthesia and surgery for 3 d. Then, the mice again were anesthetized as above and placed on a heating pad set to maintain the temperature of the mice at 37°C. A left flank incision was made, the left renal pedicle was occluded with a nontraumatic microvascular clamp, saline at 37°C was instilled in the intraperitoneal cavity, and the surgical site was covered with gauze soaked in warm saline. The mice were placed in a ventilated incubator set at 37°C. Forty minutes after placement of the clamp, the organ was allowed to reperfuse by removal of the clamp, and the wound was closed with staples. The animals then were allowed to recover from anesthesia and surgery with free access to food and water. Adoptive transfer experiments involved transfer of 20 million splenocytes from WT mice into CXCR3−/− mice a day before induction of I/R injury as described above. Sham operation was performed in a similar manner, except for clamping of the renal vessels. Renal tissues from mice were removed at 0, 6, 24, 48, and 72 h after I/R for pathologic examination and for gene expression. Blood samples were taken from the retro-orbital vein at each time point to evaluate renal function by measurement of serum creatinine levels by Modified Jaffé reaction using the Creatinine Reagent Kit (Pointe Scientific, Lincoln Park, MI).

Tissue Preparation

One third of the kidney was placed in OCT for immunohistology. One third was fixed in 10% buffered formalin followed by embedding in paraffin and staining with hematoxylin and eosin as well as periodic acid-Schiff reagent. The remaining third was snap-frozen for real-time PCR. The histologic slides of renal tissue were examined and reported in paraffin and staining with hematoxylin and eosin as well as periodic acid-Schiff reagent. The remaining third was fixed in 10% buffered formalin followed by embedding in OCT for immunohistology.

RNA Extraction and Real-Time PCR

Total RNA was extracted from kidneys with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. RNA was extracted to synthesize cDNA. Primers Express software was used to design the primers. The qPCR reaction consisted of 250 ng of cDNA, 10 μl of SYBR Green master mix (Applied Biosystems, Foster City, CA), and 250 nmol of sense and antisense primer. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured during the annealing/extension phase. The calculated number of copies was divided by the number of copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Flow Cytometry and Intracellular Cytokine Staining

All mAb were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). For intracellular cytokine staining, cells were washed in PBS that contained 2% FCS, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) according to manufacturer’s instructions, and incubated with PE-conjugated IFN-γ or isotype control mAb for 30 min at 4°C. IFN-γ production was analyzed as percentage of cytokine positivity taking into consideration the negative staining defined by staining with isotype controls.

Adoptive Transfer

We adoptively transferred 2 × 10⁷ WT CD3+ cells that were extracted from WT splenocytes with magnetic beads (Miltenyi Inc., Auburn, CA) into CXCR3−/− mice immediately after clamping. We then evaluated creatinine levels and kidney histology at 24 h.

Statistical Analyses

Data are expressed as mean ± SEM. A one-way ANOVA, followed by an unpaired t test, was performed to compare the differences between the two groups. Kaplan-Meier analysis was used for survival analysis. Statistical significance was set at P < 0.05. Analysis of data was performed using an SPSS statistical package for Windows (SPSS Inc., Chicago, IL).

Results

Temporal Expression of CXCR3 and Its Ligands

To examine the role of CXCR3 and its ligands in I/R injury, we first tested their expression in the kidney after ischemia at 6, 24, and 72 h after reperfusion. The induction of IP-10/CXCL10 and MIG/CXCL9 expression was seen as early as 6 h after clamping with a steady increase over time (Figure 1A). ITAC expression was detected at very low levels (data not shown). The induction of CXCR3 expression was also noted at 6 h after clamping; it is interesting that its expression diminished later on. CXCR3 downregulation could be explained through two pathways of rapid desensitization of CXCR3 or by internalization (Figure 1B) (32). To address whether uninephrectomy before clamping affects the contralateral kidney and contributes to the differences that we observed, we examined the expression of CXCR3 and its ligands in the contralateral kidney. No increase in the expression of CXCR3 receptor or its ligands was noted in the contralateral kidney, and the histology was also normal, without any signs of tubular injury (data not shown).

Absence of CXCR3 Provides Protection against Renal I/R Injury

To test the functional relevance of CXCR3, we induced renal I/R injury in WT and CXCR3−/− mice. WT mice showed a significant elevation of serum creatinine at 24 h (five-fold over sham) and at 72 h (six-fold over sham) after reperfusion (Figure 1C). The levels of serum creatinine for the sham and WT groups at 24 h after reperfusion was 0.58 ± 0.11 and 3.33 ± 0.80 mg/dl, respectively (P = 0.01). In the CXCR3−/− mice, the levels of serum creatinine were significantly lower than those in the WT mice at 24 and 72 h after reperfusion (Figure 1C). Although these levels were higher than those of the sham group, the difference was not statistically significant (data not shown). The survival rates of CXCR3−/− and WT groups were also compared by monitoring the viability of the mice in each group. CXCR3 deficiency was associated with a much better survival rate than those in the WT (at 10 d: WT 41.6 versus CXCR3−/− 71.7%).
78.9%; 0.01; Figure 1D). We also examined the expression of CXCR3 ligands in the CXCR3 mice after clamping. The expression of IP-10/CXCL10 increases at 6 h after clamping in the kidney of CXCR3 mice (Figure 2A). No expression of ITAC was noted. Although expression of MIG was detected at 6 h, its expression decreased at 24 h (data not shown).

Adoptive Transfer
To confirm further that CXCR3 deficiency on leukocytes was responsible for affording protection against renal I/R injury, we adoptively transferred CD3 cells that were extracted from WT splenocytes into CXCR3 mice. Transferring splenocytes restored I/R injury in CXCR3 mice, as indicated by a significant rise in the serum creatinine level (3.1 ± 0.3 mg/dl at D1 and 2.6 ± 1.0 mg/dl at D3). Creatinine levels in the adoptively transferred mice approached creatinine levels of WT mice. After adoptive transfer, CXCR3 mice showed histologic injury similar to that of regular WT mice after clamping, with a large amount of inflammation and acute tubular necrosis (ATN) (Figure 2B). As far as mortality, CXCR3 mice that received adoptively transferred WT CD3 cells showed a mortality of 50% up to 10 d, approaching that of WT mice.

Preserved Renal Histology in CXCR3 Mice
We then examined the extent of ATN at different time points after I/R. In WT mice, early stage of ATN was noted in outer medulla as early as 6 h. At 6 h, the semiquantitative score for tubular injury was 1.4 for the WT and 0.6 for the CXCR3 mice (P = 0.02). The average number of infiltrating CD3 cells per 20 high-powered fields was 12 for WT and five for the CXCR3 mice (P = 0.03).

The pathologic features of acute tubular necrosis worsened at 24 h after I/R with massive amounts of hyaline and granular casts (Figure 3A). In contrast, the degree of ATN in CXCR3 mice was markedly reduced as compared with that of WT mice at 6 and 24 h after I/R (Figure 3B). No evidence of ATN was seen in the kidneys of sham-operated animals (data not shown). At 6 h after reperfusion, immunohistology of the kidneys of WT group showed an increasing number of CD3 and macro-

Figure 1. CXC chemokine receptor 3 (CXCR3) ligands (A) and CXCR3 receptor gene expression (B) in the sham kidney and in wild-type (WT) kidney after ischemia-reperfusion (I/R; n = 4 mice per groups). WT mice show a dramatic elevation of serum creatinine at 24 h (five-fold over sham) and at 72 h (six-fold over sham) after reperfusion (C). In the CXCR3 mice, the levels of serum creatinine at 24 and 72 h after reperfusion were significantly lower than those in the WT mice (P = 0.01; D).
Preferential Recruitment of Th2 Cells in CXCR3−/− Mice
Given the importance of Th1/Th2 cytokines in the pathogenesis of I/R injury and that CXCR3 is expressed primarily by Th1 cells, we assessed the status of Th1/Th2 immune responses by evaluating intracellular cytokine staining of infiltrating cells that were extracted from the kidneys after reperfusion. The percentage of IFNγ+CD4+ cells at 6 h was much higher in the WT (79.1%) than those in CXCR3−/− (25.9%) mice (Figure 4, A and B). We also investigated the expression of Th2 cytokines, IL-4, and IL-10 between two groups. The IL-4 and IL-10 gene expression was higher in the CXCR3−/− kidneys (Figure 2, C and D).

Lower Expression of Proapoptotic/Antioxidant Genes in CXCR3−/− Mice
To explore protective mechanisms in CXCR3−/− mice, we examined and compared the change in level of expression of proapoptotic gene caspase-3 and of antioxidant genes superoxide dismutase (SOD) and heme oxygenase (HO-1) between the sham and ischemic kidneys (at 24 h) in each group. Whereas the level of caspase-3 expression was unchanged in the CXCR3−/− group, a significant increase was noted in the WT group; this was associated with marked increase in HO-1 gene expression in the CXCR3−/− group but not in the WT group (Figure 4, C through E). SOD expression was decreased in the WT group compared with no change in the CXCR3−/− group. Both HO-1 and SOD have been shown to be important in decreasing the production of ROS and attenuating ischemia (8,33).

Discussion
I/R injury is the most common cause of acute renal failure. With the increasing number of critically ill patients, I/R injury remains one of the main reasons for patients’ death in intensive care units. In transplantation, I/R injury is the primary cause of delayed graft function, which significantly contributes to morbidity and mortality in organ transplantation. Other than supportive care, there is no satisfactory therapy for this type of acute renal failure (6).

In this report, we have evaluated the role of CXCR3 receptor pathway in I/R kidney injury in mice to establish novel therapeutic strategies for I/R injury. We have studied the expression of CXCR3 and its ligands MIG/CXCL9 and IP-10/CXCL10 after induction of ischemia. Lack of CXCR3 signaling offered protection against I/R injury. Compared with the WT mice, CXCR3−/− mice had significantly lower serum creatinine levels, better survival, and significantly less histologic evidence of acute tubular necrosis. Inflammation has increasingly been recognized to contribute substantially to the pathogenesis of I/R injury.
injury. During the course of reperfusion, there is a cascade of inflammatory responses, consisting of recruitment of peripheral leukocytes and leukocyte–endothelial cell interactions (34). In this regard, Fairchild et al. (35) showed the importance of IL-8 and macrophage inflammatory protein-2 chemokines in the recruitment of neutrophils that mediate injury after ischemia. Recent studies that examined the role of T cells in the pathogenesis of I/R suggested a crucial role of T cells as the key mediators in I/R injury (14,36,37). In our study, at 6 h after reperfusion, we noted the accumulation of T cells and macrophages in the WT mice. Cellular infiltrates in the CXCR3−/− mice were less than that in WT mice at 6 h. Restoration of renal injury in CXCR3−/− mice after adoptive transfer of WT splenocytes into CXCR3−/− mice highlights the primary role of CXCR3 signaling in recruiting T cells into kidneys shortly after the induction of ischemia and mediating renal injury. The recruitment of WT CD3+ cells to the kidney in the CXCR3−/− mice after adoptive transfer may be mediated by the increase in IP-10/CXCL10 expression in the kidney. That blockade of coinhibitory pathways was also associated with attenuation of I/R injury may suggest that T and dendritic cell interactions play an important role in I/R injury (13,38,39). The damaged tubular epithelial cells were shown to express MHC class II antigens, which could be picked up and presented by recruited dendritic cells to autoreactive CD4+ T cells, signifying the importance of autoreactive cellular immunity in I/R injury (40). The other aspect of our study is to examine the importance of Th1/Th2 immune response in I/R injury. Current data suggest that the Th1 immune response could be injurious, whereas the Th2 phenotype could be protective against I/R injury (26). The proinflammatory cytokine IFN-γ (a Th1 cytokine) has been shown to generate a number of detrimental changes, including disruption of cell-matrix adhesion, inducing cell shedding into the lumen, and upregulating adhesion molecules and selectins (14,41). CD4+ T cells were reported to require IFN-γ to be able to generate injury (42). Data from the studies that used STAT6- and STAT4-deficient mice were consistent with the protective effect of Th2 T cell phenotype (26). The significance of chemokines in I/R injury could be highlighted by their importance in regulating the Th1/Th2 immune responses. Th1 cells selectively undergo chemotaxis to IP-10/ CXCL10 and MIG/ CXCL9, ligands for CXCR3 (43). The expression of chemokine receptors depends on the state of activation or differentiation of a T cell. CXCR3 and CCR5 are expressed on activated Th1 cells,
whereas CCR3, CCR4, and CCR8 expression characterizes Th2 cells. Within a few minutes after I/R, overexpression of chemokines from ischemic tissue should recruit inflammatory cells to establish early injuries. Enhanced expression of the Th1 chemokine/receptors CXCR3 will be associated with increasing monocyte/macrophage infiltrates and a Th1 response, aggravating initial injuries. Our data, consistent with previous studies, demonstrate a reduced recruitment of Th1 cells in the CXCR3−/− mice.

The release of ROS and consequent activation of apoptotic genes such as caspase-3 that occur in the reperfusion phase of I/R have been shown to be the common downstream pathway that leads to tissue injury (44–46). Injury that is caused by oxidative stress occurs when the generation of ROS exceeds the capacity of the antioxidant defenses.

In such a situation, there may be indiscriminate damage to lipids, proteins, and DNA, leading to cell dysfunction and tissue damage (8,33,47,48). Such pattern is noted in the WT group reported here. It was shown recently that specific subsets of infiltrating cells could be beneficial by overproduction of HO-1 (2). Whether there is a direct correlation between the CXCR3 signaling and such pathways is an interesting concept that could be a subject of future studies.

Despite the protection against I/R injuries in the CXCR3−/− group, we noted some mortality at early time courses after clamping, a rise in serum creatinine, and some degree of ATN in the CXCR3−/− kidneys. These highlight the probability that other chemokine/chemokine receptor pairs play a compensatory role in promoting I/R injuries. Identifying candidate chemokine receptors that are overexpressed and demonstrating their functional importance in I/R injuries are the subjects of our future studies.

**Conclusion**

Our data suggest that CXCR3-mediated recruitment of Th1 inflammatory cells could play an important role in renal injury...
that is induced by I/R. Therefore, anti-CXCR3 strategies could yield promising results in preventing the renal I/R injury.

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