Fractalkine receptor (CX3CR1) inhibition is protective against ischemic acute renal failure in mice

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ISCHEMIA IS A COMMON CAUSE of acute renal failure (ARF) (13). ARF is a life-threatening illness that continues to have a high mortality rate of 50–80% in an intensive care unit setting (13). Thus a better understanding of the pathogenesis of ARF is needed to allow interventions which would prevent the need for hemodialysis, shorten the course of ARF, and improve survival. Tubular and vascular factors, as well as inflammatory processes, are involved in the pathophysiology of ARF (13). Recently, the role of inflammation in ARF has been increasingly appreciated (4, 9, 18), with the involvement of leukocytes, adherent molecules, chemokines, and cytokines.

Endothelial injury is a feature of ischemic ARF (33). Fractalkine (CX3CL1) expression on injured endothelium functions as both a potent chemotactant and adhesive molecule for the recruitment and migration of fractalkine receptor (CX3CR1)-expressing circulating inflammatory cells into sites of inflammation (38). CX3CL1 is a major chemotactant for macrophages that express CX3CR1 (2, 23). It has been suggested that macrophages play a pathogenic role in ischemic ARF in both the rat (8, 43) and mouse (7, 20, 24). CX3CL1 is a candidate for directing macrophage infiltration in ARF. Therefore, our hypothesis is that increased CX3CL1 expression in the endothelium initiates a series of events that culminate in macrophage infiltration into the kidney and subsequent ARF and that CX3CR1 inhibition may reduce macrophage infiltration in the kidney and protect against ARF. The aim of the study in ischemic ARF in mice was to measure CX3CL1 expression, determine whether inhibition of CX3CR1 is protective against ARF, and investigate the role of macrophages.

MATERIALS AND METHODS

Ischemia protocol. Male mice (C57BL/6), aged 8–10 wk, were used (Jackson Laboratories, Bar Harbor, ME). The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Mice weighing 20–25 g were anesthetized with an injection of 2,2,2-tribromoethanol (Avertin; ip, Sigma-Aldrich, Milwaukee, WI). A midline incision was made, and both the renal pedicles were clamped for 22 min with microaneurysm clamps as previously described (31, 32). The time of ischemia was chosen to be obtainable model of ischemic ARF. Sham surgery consisted of the same surgical procedure except that clamps were not applied. Studies in ischemic ARF were performed at 24 h of postischemic reperfusion, unless otherwise stated. Blood urea nitrogen and serum creatinine were measured using an Astra Autoanalyzer (Beckman Instruments, Fullerton, CA).

CX3CR1 inhibition. Twenty-five micrograms of a polyclonal rabbit anti-rat CX3CR1 antibody (1 mg/ml, Torrey Pines Biolabs, Houston, TX) or vehicle (rabbit serum) was injected intraperitoneally 60 min before the ischemic insult. The same dosage and route of administration of the CX3CR1 antibody has been demonstrated to ameliorate cardiovascular allograft rejection (35) and crescentic glomerulonephritis (15) in mice.

Histological examination. Paraformaldehyde-fixed (4%) and paraffin-embedded kidneys were sectioned at 4 μm and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) using standard methods. Histological examinations were performed by the renal pathologist in a blinded fashion. Histological changes due to tubular necrosis in the outer stripe of the outer medulla were quantitated by counting the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilatation as follows: 0 = none, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >75%. At least 5–10 fields (×200) were reviewed for each slide.

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The number of neutrophils per high-power field (HPF: ×400) was quantitatively assessed on PAS-stained tissue by the renal pathologist. At least 10 fields were counted in the outer stripe of the outer medulla for each slide.

Morphological criteria were used to count apoptotic tubular cells on PAS-stained tissue. Morphological characteristics included cellular rounding and shrinkage, nuclear chromatin condensation, and formation of apoptotic bodies. Apoptotic tubular cells were quantitatively assessed per 10 HPF in the outer stripe of the outer medulla by the renal pathologist in a blinded fashion. At least 10 fields were counted for each slide.

Endothelial cells in culture. MS1 (MILE SVEN 1) mouse endothelial pancreatic islet cells were purchased from American Type Culture Collection (catalog no. CRL-2279, ATCC, Manassas, VA). The line retains the properties of endothelial cells including uptake of acetylated LDL and expression of both factor VIII-related antigen and VEGFR-2 in MS1 microvascular endothelial cells (data not shown). Cells were grown in DMEM. Culture Collection (catalog no. CRL-2279, ATCC, Manassas, VA).

Western blot analysis. Whole kidney was homogenized in RIPA buffer plus proteinase inhibitors and immunoblotted as previously described (12). An polyclonal anti-rat CX3CL1 antibody produced in goats that recognizes full-length mouse CX3CL1 (100 kDa) was used (catalog no. AF-537, R&D Systems, Morrisville, NC). Recombinant mouse fractalkine which lacks 57 COOH-terminal amino acids (85 kDa) was used as a positive control. A rabbit polyclonal antibody against mousefractalkine which lacks 57 COOH-terminal amino acids (85 kDa) was used as a loading control (catalog no. sc-10731, Santa Cruz Biotechnology, Santa Cruz, CA). Images for densitometry were analyzed using 1D Image Software (Kodak Digital Science, Rochester, NY).

Immunofluorescence studies. Kidney tissues were embedded in OCT, snap-frozen in liquid nitrogen, and stored at −80°C until sectioning. Cryostat sections (5 μm) were fixed in 70% acetone/30% methanol and prepared for immunofluorescence studies as previously described (12). Primary antibodies used were CX3CL1, the same antibody as described for immunoblotting, and a rat anti-mouse CD11b monoclonal antibody (catalogue no. MCA74, Serotec, Oxford, UK).

The intensity of endothelial staining of CX3CL1 in arteries was scored from 0 to 3+. Representative pictures of the grading scale (0–3+) are demonstrated in Fig. 2A. More than five blood vessels were counted in each kidney by a blinded observer.

Macrophage depletion studies. Liposomes (vehicle) and liposome-encapsulated clodronate (LEC; Roche Diagnostics, Mannheim, Germany) were prepared as previously described in detail (39–41). Briefly, macrophages phagocytose the liposomes, resulting in the release of clodronate into the cytoplasm and death of the macrophage. Empty liposomes, not containing clodronate, and prepared under exactly the same conditions as the LEC, were used as a control. Mice received a tail vein injection of 100 μl of empty liposomes or LEC at 6 and 2 days before induction of ischemia by bilateral renal pedicle clamp.

At 24 h of post-ischemic reperfusion, spleens were prepared for FACScan analysis as we have previously described (14). Staining was detected by a Beckton-Dickinson FACScan (BD Immunocytometry Systems, San Jose, CA) flow cytometer. CellQuest Software (BD Immunocytometry Systems) was used to analyze flow cytometric data.

Statistical analysis. Nonnormally distributed data were analyzed by the nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using ANOVA with a Newman-Keuls post-test. A P value of <0.05 was considered statistically significant. Values are expressed as means ± SE.
Anti-CX3CR1 antibody. Administration of the anti-CX3CR1 antibody (25 μg ip 1 h before induction of ischemia) protected against ARF, as determined by renal function and histology. Serum creatinine (mg/dl) was 0.2 ± 0.03 in sham-operated mice, 2.1 ± 0.2 in vehicle-treated ARF (P < 0.001 vs. sham, n = 12), and 1.5 ± 0.2 in ischemic ARF mice treated with the anti-CX3CR1 antibody (P < 0.05 vs. vehicle-treated ischemic ARF) (Fig. 4A). Blood urea nitrogen (mg/dl) was 24 ± 2 in
Administration of LEC in ischemic ARF had no significant effect on apoptosis of tubular cells. The number of apoptotic tubular cells per 10 HPF was zero in sham, 11.6 ± 0.4 in vehicle-treated ARF ($P < 0.05$ vs. sham), and 9.6 ± 0.5 in LEC-treated ARF ($P < 0.05$ vs. sham, $P > 0.05$ vs. vehicle-treated ARF).

**DISCUSSION**

Evidence is accumulating regarding the pathophysiological effects of CX3CL1 and its ligand CX3CR1 in the pathogenesis of various inflammatory diseases such as atherosclerosis (29), allograft rejection (22), human immunodeficiency virus infection (17), and rheumatoid arthritis (42). In renal disease, CX3CL1 expression is increased in patients with renal tubulointerstitial inflammation, with the strongest expression localized to vascular sites near to macrophage inflammation (6).

sham-operated mice, 129 ± 6 in vehicle-treated ARF ($P < 0.001$ vs. sham, $n = 12$), and 93 ± 5 in ischemic ARF mice treated with the anti-CX3CR1 antibody ($P < 0.05$ vs. ischemic ARF). The acute tubular necrosis (ATN) score was 0.6 ± 0.3 in sham, 4.8 ± 0.2 in vehicle-treated ARF ($P < 0.01$ vs. sham), and 4.0 ± 0.2 in CX3CR1 antibody-treated ARF ($P < 0.05$ vs. ARF) (Fig. 4B).

Administration of the anti-CX3CR1 antibody in ischemic ARF had no effect on apoptosis of tubular cells. The number of apoptotic tubular cells per 10 HPF was zero in sham, 13 ± 6 in vehicle-treated ARF ($P < 0.05$ vs. sham), and 13 ± 4 in CX3CR1 antibody-treated ARF ($P < 0.05$ vs. sham, $P > 0.05$ vs. vehicle-treated ARF) (Fig. 4C).

**Immunofluorescence staining for CD 11b-positive macrophages.** CX3CL1 is expressed on activated endothelial cells and is both a chemoattractant and an adhesion molecule for inflammatory cells carrying the fractalkine receptor CX3CR1. To elucidate the mechanism of protection by anti-CX3CR1 antibody treatment, it was determined whether CX3CR1 inhibition reduced macrophage infiltration in the kidney in ARF.

Anti-CX3CR1 antibody treatment significantly reduced the number of CD11b-positive macrophages in the kidney during ischemic ARF, as judged by immunofluorescence staining. The number of CD11b (+) cells per HPF (×400) in the outer stripe of the outer medulla was 0.7 ± 0.5 in sham, 15.7 ± 1.1 in vehicle-treated ARF ($P < 0.01$ vs. sham), and 11.7 ± 1.5 in CX3CR1 antibody-treated ARF ($P < 0.05$ vs. vehicle-treated ARF) (Fig. 5A). Representative pictures of the immunofluorescence staining for CD11b-positive cells in the kidney in ischemic ARF are demonstrated in Fig. 5, B–D.

CX3CR1 is expressed on monocyte/macrophages and NK cells rather than neutrophils (23). The CX3CR1 antibody had no effect on neutrophil infiltration in the kidney in ischemic ARF. Neutrophils per HPF was 0.0 ± 0.0 in sham, 99 ± 51 in vehicle-treated ischemic ARF ($P < 0.05$ vs. sham), and 91 ± 21 in CX3CR1 antibody-treated ischemic ARF ($P < 0.05$ vs. sham, $P > 0.05$ vs. vehicle-treated).

**Macrophage depletion studies.** Administration of LEC resulted in a 70% reduction in macrophages in the spleen (data not shown) and attenuated the increase in macrophage infiltration in the kidney in ischemic ARF (Fig. 6, A–D). Macrophage depletion with LEC protected against ARF, as determined by renal function and the ATN score (Fig. 7, A and B).
Fig. 6. Macrophage depletion studies. A: liposomal-encapsulated clodronate (LEC) resulted in a significant decrease in macrophage infiltration in the kidney in ischemic ARF. *P < 0.01 vs. sham. **P < 0.05 vs. ischemic ARF/vehicle. Empty liposomes prepared in the same manner as LEC were used as the vehicle. Representative pictures of immunofluorescence staining for CD11b-positive cells (arrows) in the outer stripe of outer medulla in sham-operated (B), ischemic ARF/vehicle (C), and ischemic ARF/Ab (D) are demonstrated.

Fig. 5. Immunofluorescence staining for CD11b. A: the number of CD11b (+) macrophages was significantly increased in ischemic ARF compared with sham (*P < 0.001 vs. sham) and reduced by anti-CX3CR1 antibody treatment (***P < 0.01 vs. vehicle). B: representative pictures of immunofluorescence staining for CD11b-positive cells (arrows) in the outer stripe of outer medulla in sham-operated (B), ischemic ARF/vehicle (C), and ischemic ARF/Ab (D) are demonstrated.
CX3CR1-positive cells were seen in human kidney biopsy specimens in a variety of conditions including kidney tumors, renal transplant nephrectomies, renal transplant biopsies, and glomerulonephritis. Also, a recent report demonstrated that crescentic glomerulonephritis in the rat was prevented by CX3CR1 antibody administration. Furuichi et al. have recently demonstrated that crescentic glomerulonephritis in the rat was prevented by CX3CR1 antibody inhibition. CX3CL1 has a mucin-like stalk that extends the chemokine domain away from the endothelial cell surface, enabling presentation of the CX3C-chemokine domain to leukocytes. Expression of CX3CL1 enables bypassing of the first two steps of the adhesion cascade (i.e., rolling and triggering) and mediates cell adhesion between circulating leukocytes and endothelial cells as well as extravasation of these cells into the interstitium of the kidney. CX3CL1 has a major chemotactic property for these cells but not neutrophils. CX3CL1 may play a major role in the pathogenesis of ischemic ARF.
macrophage depletion with LEC resulted in functional and histological protection against ischemic ARF, confirming the role of macrophages in ischemic ARF.

Treatment of brain microglia cells in vitro with fractalkine inhibits fas ligand-mediated apoptosis (3). Macrophages play an important role in the clearance of apoptotic cells (28). The effect of CX3CR1 inhibition or inhibition of macrophage infiltration in the kidney on renal tubular cell apoptosis is not known. In the present study, administration of the CX3CR1 antibody protected against ATN but had no effect on tubular cell apoptosis. This suggests that the protective effect of the CX3CR1 inhibition in early ischemic ARF is mediated by inhibition of tubular cell necrosis rather than inhibition of tubular cell apoptosis. In addition, inhibition of macrophage infiltration in the kidney by LEC protected against ATN but had no effect on tubular cell apoptosis. This suggests that the protective effect of macrophage depletion by LEC in ischemic ARF is mediated by inhibition of tubular cell necrosis rather than inhibition of tubular cell apoptosis.

The expression of membrane-bound CX3CL1 can be markedly induced on primary endothelial cells by inflammatory cytokines, such as TNF-α, IL-1, and IFN-γ (1). In this regard, TNF-α expression is increased in ischemic ARF (27), and inhibition of TNF-α attenuates ischemia-induced renal tubular apoptosis (30). IFN-γ produced by T cells may play a pathogenic role in ischemic ARF in mice (5). However, inhibition of IL-1, an inducer of CX3CL1, is not protective against ischemic ARF (21). Investigation of the proinflammatory cytokine that increases CX3CL1 expression in ischemic ARF merits further study.

Acute allograft rejection is characterized by an intense cellular immune response marked by the influx of circulating leukocytes into the transplant kidney (34). CX3CL1 expression in vascular endothelium is significantly enhanced in rejecting cardiac allografts, and treatment with anti-CX3CR1-blocking antibodies significantly prolonged allograft survival (35). In addition, the survival time of allogeneic cardiac transplants is significantly increased in the presence of subtherapeutic levels of cyclosporin A in CX3CR1 knockout mice associated with a reduction in the infiltration of macrophages and NK cells (22). These studies and our results that CX3CR1 inhibition is protective in ischemic ARF indicate a potential future therapeutic role of CX3CR1 inhibition.

In summary, in ischemic ARF, there is upregulation of CX3CL1 protein in the kidney, specifically in blood vessels. In ischemic ARF, CX3CR1 inhibition using a specific antibody is partially protective, and CX3CR1 inhibition is associated with reduced macrophage infiltration in the kidney. To confirm an injurious role of macrophages in ischemic ARF, depletion of macrophages in the kidney is protective against ischemic ARF. In conclusion, CX3CR1 inhibition is protective against ischemic ARF, perhaps by reducing macrophage infiltration in the kidney.

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