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

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Fractalkine receptor (CX3CR1) inhibition is protective against ischemic acute renal failure in mice. Am J Physiol Renal Physiol 294: F264–F271, 2008. First published November 14, 2007; doi:10.1152/ajprenal.00204.2007.—Fractalkine (CX3CL1) is expressed on injured endothelial cells and is a potent chemoattractant and adhesion molecule for macrophages carrying the fractalkine receptor (CX3CR1). The aim of this study was to investigate the role of CX3CL1, and its ligand CX3CR1, in ischemic acute renal failure (ARF) in mice. On immunoblotting, CX3CL1 protein expression in the kidney increased markedly in ischemic ARF. On immunofluorescence staining, the intensity of CX3CL1 staining in blood vessels was significantly more prominent in ischemic ARF compared with controls. A specific anti-CX3CR1 antibody (25 μg ip 1 h before induction of ischemia) was functionally and histologically protective against ischemic ARF. CX3CR1 is predominantly expressed on macrophages. Macrophage infiltration in the kidney in ischemic ARF was significantly decreased after anti-CX3CR1 antibody treatment. To determine the role of macrophages in ischemic ARF, macrophages in the kidney were depleted using liposomal-encapsulated clodronate (LEC). LEC resulted in significant functional and histological protection against ischemic ARF. In summary, in ischemic ARF, 1) there is upregulation of CX3CL1 protein in the kidney, specifically in blood vessels; 2) CX3CR1 inhibition using a specific antibody is partially protective and is associated with reduced macrophage infiltration in the kidney; and 3) macrophage depletion in the kidney is protective.

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 obtain a reversible 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 Bioslabs, 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 cardiac 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 ischemic ARF were determined 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 >76%. 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 the VEGF receptor (VEGFR). The expression of VEGFR-1 and VEGFR-2 in MS1 microvascular endothelial cells (data not shown) was confirmed by Western blot analysis. Cells were grown in DMEM.

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**Western blot analysis.** Whole kidney was homogenized in RIPA buffer plus protease 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 amino acids 76–375 of actin of human origin (43 kDa) was used as a positive control. A rabbit polyclonal antibody (catalog no. AF-537, R&D Systems, Morrisville, NC). Recombinant goats that recognizes full-length mouse CX3CL1 (100 kDa) was used for CX3CL1. There was an upregulation of CX3CL1 protein expression in cells incubated with antimycin A (0.025 μM for 6 h) compared with vehicle-treated cells.

**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).

**Immunofluorescence staining for CX3CL1.** CX3CL1 is predominantly expressed in endothelial cells. To confirm the endothelial location of CX3CL1 staining in the kidney, immunofluorescence was performed. Using the grading scale described in MATERIALS AND METHODS (Fig. 2A), the endothelial staining score was 1.4 ± 0.2 in sham-operated mice and 2.4 ± 0.2 in ischemic ARF (P < 0.05; n = 4) (Fig. 2B). Representative pictures of CX3CL1 staining in capillaries in the outer stripe of the outer medulla in sham-operated and ARF mice are demonstrated in Fig. 2. C and D, respectively. Representative pictures of CX3CL1 staining in capillaries in a normal glomerulus from a sham-operated mouse is demonstrated in Fig. 2E.

**Endothelial cells.** To determine whether chemical hypoxia has a direct effect on endothelium, mouse microvascular endothelial cells (MS1 cells) were treated with antimycin A (as described in MATERIALS AND METHODS) and then immunoblotted for CX3CL1. There was an upregulation of CX3CL1 protein expression in cells incubated with antimycin A (0.025 μM for 6 h) compared with vehicle-treated cells.

**Immunoblotting of CX3CL1.** The protein expression of CX3CL1 in whole kidney at 24 h post-ischemic reperfusion was increased >10-fold in ischemic ARF mice compared with sham-operated mice (Fig. 1A). A time course of CX3CL1 protein expression in ischemic ARF demonstrated that the increased expression of CX3CL1 was prominent at 8 h of post-ischemic reperfusion (Fig. 1A). Densitometric analysis is demonstrated in Fig. 1B.

**Results.**

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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 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, 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).

Fig. 3. Endothelial cells. Mouse microvascular endothelial cells (MS1 cells) were treated with antimycin A (AA; 0.025 μM for 6 h) and then immunoblotted for CX3CL1 (100 kDa). There was an upregulation of CX3CL1 protein expression in cells incubated with AA compared with vehicle-treated cells (Veh). Recombinant mouse CX3CL1, which lacks 57 COOH-terminal amino acids (85 kDa), was used as a positive control (Pos). Actin (43 kDa) was used as a loading control. Representative immunoblot of at least 3 separate experiments is shown.

Fig. 4. Administration of anti-CX3CR1 antibody. A and B: administration of the anti-CX3CR1 antibody (Ab) before induction of ischemia protected against ischemic ARF, as determined by serum creatinine (*P < 0.001 vs. sham, **P < 0.05 vs. vehicle-treated ARF). C: antibody had no effect on apoptosis of tubular cells (*P < 0.05 vs. sham, **P > 0.05 vs. vehicle-treated ARF).
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.

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 the 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 (36). Also, a recent report demonstrated that crescentic glomerulonephritis in the rat was prevented by CX3CR1 antibody administration (15). Furuichi et al. (19) have recently demonstrated, using a 60-min bilateral renal pedicle clamp model in mice, that CX3CR1 in part regulates renal interstitial fibrosis and renal failure at 7–14 days of post-ischemic reperfusion. However, the results of the Furuichi study at 24 h of post-ischemic reperfusion show very important differences compared with our study. 1) In the Furuichi study, serum creatinine and ATN scores were not different between wild-type and fractalkine receptor-deficient mice at any time point. In our study, serum creatinine and ATN scores were attenuated by CX3CR1 antibody inhibition at 24 h. 2) In the Furuichi study, fractalkine protein level and spatial distribution changed at 24–48 h. In our study, fractalkine protein expression increased as early as 8 h. 2) In the Furuichi study, serum creatinine and ATN scores were not different between wild-type and fractalkine receptor-deficient mice at any time point. In our study, serum creatinine and ATN scores were attenuated by CX3CR1 antibody inhibition at 24 h. 3) In the Furuichi study, macrophage (F4/80) cell number was not different between wild-type and fractalkine receptor-deficient mice. In our study, macrophage infiltration (CD11b) was attenuated by CX3CR1 antibody inhibition. 4) In the discussion section of their study, Furuichi et al. conclude that early ischemic ATN is not a CX3CR1-dependent process. In our study, we conclude that early ischemic ATN is partially a CX3CR1-dependent process as serum creatinine, ATN score, and macrophage infiltration are reduced by CX3CR1 inhibition at 24 h of post-ischemic reperfusion. The role of CX3CL1 and its ligand CX3CR1 and the effect of CX3CR1 antibody administration in the early "initiation" phase of ischemic ARF are the subjects of the present study.

CX3CL1 is expressed on injured endothelial cells (38). The rationale for studying CX3CL1 in ARF is that endothelial injury is a feature of ischemic ARF. In ischemic ARF in rodents, microvascular endothelial injury and dysfunction as well as an increase in circulating von Willebrand factor (vWF) have been demonstrated (37). It has been suggested that endothelial injury and dysfunction play an important role in the extension phase of ischemic ARF (33). In the present study, we demonstrate that expression of CX3CL1, a marker of endothelial injury, is increased in the kidney, specifically in blood vessels, in ischemic ARF. In addition, our in vitro data demonstrating an increase in CX3CL1 expression in cultured endothelial cells treated with antimycin A support the evidence that ischemia increases CX3CL1 expression on injured endothelial cells.

Endothelial injury may initiate an inflammatory response in the kidney in ischemic ARF (9). The inflammatory response may play a major role in the pathogenesis of ischemic ARF (4, 18). CX3CR1 is expressed on monocyte/macrophages, NK cells, and some CD8+ T cells (38), and CX3CL1 is a major chemoattractant for these cells but not neutrophils (2). 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 is one of the candidates for directing macrophage infiltration in ischemic ARF. However, other adhesion molecules and chemokines are known to play an injurious role in ischemic ARF. It has been suggested that ICAM-1 inhibition is protective in ischemic ARF (25, 26) and VCAM-1 expression is increased in cold ischemic kidneys (10, 11). Also, it has recently been demonstrated that blockade of the CXC chemokine receptor 3 (CXCR3) reduces inflammation and protects against ischemic ARF (16). In the present study, CX3CR1 inhibition decreased macrophage infiltration, but not neutrophil infiltration, in the kidney. However, compared with clodronate, the effects of anti-CX3CR1 on macrophage infiltration were modest. In addition, the functional and histological protection by CX3CR1 inhibition was partial, highlighting the potential role of other adhesion molecules and chemokines, for example, ICAM-1, VCAM-1, and CXCR3 in macrophage infiltration in the kidney in ischemic ARF.

Our published data indicate that neither neutrophils (32) nor CD4+ T cells (14) play a pathogenic role in ischemic ARF. It has been suggested that macrophages play a pathogenic role in ischemic ARF, as significant monocyte/macrophage adhesion and infiltration occur in the outer stripe of the outer medulla as early as 24 h post-ischemic reperfusion in the rat (8, 43) and mouse (7, 20, 24). In addition, specific macrophage depletion is protective against ischemic ARF in mice (7, 24), and gene therapy in rats expressing a nonfunctional NH2-terminal-truncated monocyte chemoattractant protein-1 (MCP-1) reduced macrophage infiltration and ATN (20). In the present study,
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 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 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 rejecting cardiac allografts, and treatment with anti-CX3CR1-blocking antibodies significantly prolonged allograft survival (35).

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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