CCR2 Signaling Contributes to Ischemia-Reperfusion Injury in Kidney

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Abstract. Examined were CCR2-deficient mice to clarify the contribution of macrophages via monocyte chemoattractant protein 1 (MCP-1 or CCL2)/CCR2 signaling to the pathogenesis of renal ischemia-reperfusion injury. Also evaluated was the therapeutic effects via the inhibition of MCP-1/CCR2 signaling with propagermanium (3-oxygermylpropionic acid polymer) and RS-504393. Renal artery and vein of the left kidney were occluded with a vascular clamp for 60 min. A large number of infiltrated cells and marked acute tubular necrosis in outer medulla after renal ischemia-reperfusion injury was observed. Ischemia-reperfusion induced the expression of MCP-1 mRNA and protein in injured kidneys, followed by CCR2-positive macrophages in interstitium in wild-type mice. The expression of MCP-1 was decreased in CCR2-deficient mice compared with wild-type mice. The number of interstitial infiltrated macrophages was markedly smaller in the CCR2-deficient mice after ischemia-reperfusion. CCR2-deficient mice decreased the number of interstitial inducible nitric oxide synthase–positive cells after ischemia-reperfusion. The area of tubular necrosis in CCR2-deficient mice was significantly lower than that of wild-type mice after ischemia-reperfusion. In addition, CCR2-deficient mice diminished KC, macrophage inflammatory protein 2, epithelial cell-derived neutrophil-activating peptide 78, and neutrophil-activating peptide 2 expression compared with wild-type mice accompanied with the reduction of interstitial granulocyte infiltration. Similarly, propagermanium and RS-504393 reduced the number of interstitial infiltrated cells and tubular necrosis up to 96 h after ischemia-reperfusion injury. These results revealed that MCP-1 via CCR2 signaling plays a key role in the pathogenesis of renal ischemia-reperfusion injury through infiltration and activation of macrophages, and it offers a therapeutic target for ischemia-reperfusion.

Leukocytic infiltration plays a critical role in ischemia-reperfusion injury, such as cerebrovascular accident, myocardial ischemia, and ischemic acute renal failure. Concerning renal diseases, ischemia-reperfusion is important in renal transplantation, shock state by various reasons, or renal artery stenosis (1). Ischemia-reperfusion injury in kidney is pathologically characterized by tubular epithelial cell necrosis and/or apoptosis with marked cell infiltration (2–4). Various types of inflammatory cells have been reported to participate in the pathogenesis of renal injury after ischemia-reperfusion (1,4). Nevertheless, most studies on renal ischemia-reperfusion injury have focused on the infiltration and activation of granulocytes (2,3,5). Macrophages infiltrate in the postischemic kidneys (6) and are supposed to play a role via the production of cytokines and chemokines (7,8). However, precise roles of macrophages in the pathogenesis of renal ischemia-reperfusion injury remain to be fully evaluated.

Monocyte chemoattractant protein 1 (MCP-1; also termed monocyte chemotactic and activating factor, or CCL2), a well known member of the CC family of chemokines, is reported to be the key molecule in terms of chemotaxis and activation of macrophages (9). In renal diseases, MCP-1 has been extensively involved in tubulointerstitial damage, including cell infiltration and interstitial fibrosis of chronic rejection of renal transplantation, IgA nephropathy, crescentic glomerulonephritis, and diabetic nephropathy (10–14). CCR2, a cognate recep-

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tor for MCP-1 (15,16), is a seven-transmembrane-spanning chemokine receptor, and recent studies revealed that MCP-1/CCR2 signaling is involved in human crescentic glomerulonephritis (13,17) and tubulointerstitial damage induced by unilateral ureteral tract obstruction (18). However, precise roles of MCP-1 and CCR2 in interstitial infiltration and activation of macrophages in ischemia-reperfusion injury remain to be investigated.

In this study, we tested the hypothesis that MCP-1/CCR2 signaling is required for macrophage infiltration and activation in ischemia-reperfusion injury. To achieve this goal, we evaluated renal ischemia-reperfusion injury in CCR2-deficient mice. In addition, we further evaluated the therapeutic effects of propagermanium and RS-504393. Propagermanium is an organic germanium compound with a chemical structure of $[(0_{1/2})_3GeCH_2CH_2CH_2H]_n$, 3-oxygermylpropionic acid polymer and a molecular weight of $(9.29 \pm 5.72) \times 10^4$. Propagermanium targets glycosylphosphatidylinositol-anchored proteins that are closely associated with CCR2, and selectively inhibit MCP-1/CCR2 signaling (20). RS-504393 is a specific small organic molecule antagonist of CCR2. This compound is not a chemotaxis agonist and does not stimulate postreceptor signaling of any kind. The compound specifically inhibits MCP-1 and MCP-3 signaling through CCR2; however, this is not an antagonist of CXCR1, CCR1, or CCR3. This antagonist inhibits the receptor by occupation of a binding site that includes acidic residue Glu^{291} (21).

We now report that CCR2-deficient mice were protected from acute tubular necrosis and cell infiltration. In addition, the number of granulocytes, some of which expressed CCR2, reduced in diseased kidney in accordance with diminished CXC chemokine expression. Furthermore, propagermanium also reduced the number of interstitial infiltrated cells and tubular necrosis to similar levels as those observed in CCR2-deficient mice. Thus, CCR2 signaling contributes to renal ischemia-reperfusion injury via the infiltration and activation of macrophages and thereby may offer the therapeutic target for ischemia-reperfusion.

Materials and Methods

Chemotaxis Assay

Murine spleen was removed from 7-wk-old ICR mouse (Clea Japan, Tokyo, Japan). Spleen cells were dispersed in PBS and suspended in RPMI 1640 medium (Life Technologies Laboratories, Grand Island, NY) supplemented with 0.5% BSA and 25 mM Hepes pH 7.4. Cells were incubated with the indicated concentrations of RS-504393 for 20 min and then applied to the chemotaxis assay.

Migration of murine spleen cells by murine MCP-1 and macrophage inflammatory protein 1α (MIP-1α) was assayed in a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) and 5-μm-pore-size polyvinylpyrrolidone-free polycarbonate filter membranes (Neuro Probe). Twenty-six microliters of the medium (RPMI 1640 medium supplemented with 0.5% BSA, 25 mM HEPES, pH 7.4) containing 1.25 nM murine MCP-1 (R&D Systems, Minneapolis, MN) or MIP-1α were (R&D Systems) added to the lower chamber. The filter membrane was sandwiched between lower and upper chambers, and 50 μl of spleen cell suspension (2×10^6 cells/ml) was added to the upper chamber. After incubation for 2 h, the migrated cells on the filter membrane were fixed, stained with Diff-Quik solution (International Reagents, Kobe, Japan), and counted by microscopic observation in high-power fields ($\times$600).

Animals

Mice deficient in the expression of CCR2 were generated by the process of gene targeting in murine embryonic stem cells (19), and a breeding colony was maintained under specific-pathogen-free conditions. The male CCR2-deficient and wild-type control animals were on an outbred C57BL/6J $\times$ 129/Ola genetic background (more than eight generations) and were used at 8 wk of age. All procedures used in the animal experiments complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals in Takaramachi Campus of Kanazawa University.

Renal Ischemic Model

CCR2-deficient and wild-type mice were anesthetized with diethyl ether and pentobarbital sodium. The flank incision was made, and the renal artery and vein of the left kidney were occluded with a vascular clamp for 60 min. The clamp was then removed, and the organ was allowed to reperfuse. After the clamp was released, the frank incision was closed in two layers with 4-0 silk sutures. The animals received warm saline instilled into the peritoneal cavity during the procedure and were then allowed to recover with free access to food and water. Sham operation was performed in a similar manner, except for clamping the renal vessels. Zero, 4, 24, or 48 h after ischemia-reperfusion, renal tissues from five mice at each time point were removed for pathologic examination. Blood samples were taken from the abdominal aorta at each time point to evaluate renal function. Blood urea nitrogen (BUN) was measured by standard urease assays.

To evaluate the postnatal inhibition of MCP-1/CCR2 signaling, we determined the therapeutic effects of propagermanium and RS-504393. Mice received propagermanium (8 mg/g orally) from 8 d before ischemia until the day the animal was killed. Mice also received RS-504393 (2 mg/kg orally) from the day of ischemia every 12 h until the day the animal was killed. The left kidneys of propagermanium and RS-504393–treated mice were occulted with a vascular clamp for 60 min and killed 4, 24, 48, or 96 h after ischemia-reperfusion for pathologic examination.

Tissue Preparation

One portion of the renal tissue was fixed in 10% buffered formalin followed by embedding in paraffin and staining with hematoxylin and eosin as well as periodic acid–Schiff reagent. Two independent observers with no knowledge of the experimental design evaluated each section. Total glomerular cell numbers were measured at least 50 glomeruli for each mouse and expressed as the number per glomerular cross section. The number of interstitial infiltrated cells was counted in 20 randomly selected high-power fields ($\times$400) of outer medulla. Tubular necrosis was evaluated by determining the percentage of tubules in the outer medulla in which epithelial necrosis or necrotic debris was observed. However, the tubuli with repaired tubular epithelial cells were not approved as the necrotic area. The percentage of area of tubular necrosis in outer medulla of diseased kidney was evaluated by NIH image. A part of each specimen was prefixed with 2.5% glutaraldehyde and postfixed with 4% osmic acid, progressively dehydrated in an alcohol series, embedded in Epok 812, and cut into ultrathin sections. These were double-stained with uranyl acetate and lead citrate and were examined by electron microscopy (Hitachi H-600, Tokyo, Japan).

Immunohistochemical Studies

The other portion of fresh renal tissue, embedded in OCT compound and snap-frozen in *n*-hexane cooled with a mixture of dry ice and acetone, were cut at 6 μm on a Cryostat (Tissue-Tek systems; Miles, Naperville, IL). The presence of F4/80-positive macrophages and granulocytes was detected immunohistochemically by rat anti-mouse F4/80 monoclonal antibody (clone A3-1; BMA Biomedicals, Augst, Switzerland) or rat anti-mouse granulocyte monoclonal antibody (clone RB6-8C5; Leinco Technologies, MO). The presence of inducible nitric oxide synthetase (iNOS) was detected immunohistochemically by rabbit anti-murine iNOS antibodies (Wako Pure Chemicals Industries, Osaka, Japan). The number of interstitial infiltrated F4/80-positive macrophages, granulocytes, and iNOS-positive cells was counted in 20 randomly selected high-power fields ($\times$400) of outer medulla, where cell migration was maximal. Two observers independently examined the immunohistochemical findings without knowledge of the experimental design.

MCP-1 Expression

The presence of MCP-1 protein was demonstrated immunohistochemically in tissue specimens of ischemia-reperfusion kidney by the indirect avidin-biotinylated peroxidase complex method with goat anti-mouse MCP-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (22). The antigen was retrieved with Target Retrieval Solution (DAKO, Carpinteria, CA). To evaluate the specificity of these antibodies, tissue specimens were stained with the absorbed antibodies with excess amount of the blocking peptide (Santa Cruz Biotechnology). To determine the expression of MCP-1 mRNA, total RNA was extracted from the whole kidneys from five mice in each group. cDNA was reverse-transcripted from 5 μg total RNA and combined from five mice in each group (1 μg RNA per mouse) with SuperScript II RNase H^- reverse transcriptase (InVitrogen, San Diego, CA). Reverse transcription was performed by the following parameters: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. For all PCR experiments, the LightCycler (Roche Diagnostics, Basel, Switzerland) was used. Subsequently, real-time reverse transcriptase–PCR (RT-PCR) was performed in the LightCycler using the LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics). Primers for MCP-1 (forward: 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3', reverse: 5'-TTCCTTCTTGGGGTCAGCACAGAC-3') (23) and 2 μl of the cDNA prepared above were used to detect MCP-1. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 10 s at 55°C, and 20 s at 72°C. MCP-1 mRNA expression in each sample was finally described as copies/μg·total RNA. The expression of MCP-1 in each sample was quantitated in separate wells. No PCR products were detected in the real-time RT-PCR procedure without reverse transcription, indicating that the contamination of genomic DNA was negligible. Gels of the PCR products after quantification of MCP-1 were negligible. Gels of the PCR products after quantification of MCP-1 by real-time RT-PCR showed a single band (270 bp) with the expected size (data not shown).

Dual Staining

To identify the CCR2-positive cells, we used a dual-labeled color immunofluorescence immunohistochemistry. Briefly, sections were first incubated with goat anti-mouse CCR2 antibodies (clone sc-6228; Santa Cruz Biotechnology) overnight. After rinsing in PBS, the rat anti-mouse F4/80 antibody (clone A3-1; BMA Biomedicals) or rat anti-mouse granulocyte antibody (clone BR6-8C5; Leinco Technologies) was added and the sections incubated overnight. To evaluate the specificity of goat anti-mouse CCR2 antibodies, tissue specimens were stained with the absorbed antibodies with excess amount of CCR2 protein (sc-6228P; Santa Cruz Biotechnology) and goat IgG as a negative control. The CCR2 staining was visualized by incubating the sections for 120 min with FITC-conjugated donkey anti-goat IgG antibodies (1:200; Jackson Immunoresearch Laboratory, West Grove, PA). After rinsing, the sections were incubated for 120 min with Cy3-conjugated donkey anti-rat IgG antibodies (1:200; Jackson Immunoresearch Laboratory) to visualize F4/80 or granulocytes.

Adobe Photoshop software was used for image handling, and the three-color channels were handled separately. Moreover, to evaluate the relation between MCP-1– and F4/80-positive cells, we used a dual-labeled color immunofluorescence immunohistochemistry with rabbit anti-mouse MCP-1 antibodies and rat anti-mouse F4/80 antibody. Briefly, Vectastain ABC-alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) was used to detect MCP-1. Then the slides were blocked by 0.1% sodium azide and 0.3% H_2O_2 in methanol for 15 min. After this process, the Vectastain ABC-peroxidase kit (Vector Laboratories) was used to detect F4/80.

Detection of KC, MIP-2, Epithelial Cell–Derived Neutrophil-Activating Peptide 78, and Neutrophil-Activating Peptide 2 Transcripts in Diseased Kidneys

To determine the renal transcripts of KC, MIP-2, epithelial cell–derived neutrophil-activating peptide 78 (ENA-78), and neutrophil-activating peptide 2 (NAP-2), total RNA was extracted from the whole kidneys from five mice in each group after ischemia-reperfusion to perform RT-PCR. cDNA was reverse transcripted from 5 μg total RNA and combined from five mice in each group (1 μg RNA per a mouse) by using a RT-PCR kit (Takara Shuzo, Tokyo, Japan). The cDNA product was amplified by PCR. Primers for KC (5' primer TGCACCCAAACCGAAGTCATAG; 3' primer GTGGTTGACACTTAGTGGTCTC) (24), MIP-2 (5' primer GGCACATCAGGTACGATCCAG; 3' primer ACCCTGCCAAGGGTTGACTTC) (25), ENA-78 (5' primer CTCAGTCATAGCCGCAACCGAGC; 3' primer CCGTTCTTTCCACTGCGAGTGC) (26), or NAP-2 (5' primer GTTGCAGAGGTTGCTTG; 3 'primer ACATCCCTTCCCCTTTC) (27) were used to detect KC, MIP-2, ENA-78, or NAP-2 transcripts. The housekeeping gene *GAPDH* was used for PCR controls. Ten microliters of PCR products were run on 2.0% agarose gel and stained with ethidium bromide; then gene-specific bands were visualized under ultraviolet light.

Statistical Analyses

The mean and SEM were calculated on all of the parameters determined in this study. Statistical analyses were performed by Wilcoxon rank-sum test, unpaired *t* test, Kruskal-Wallis test, and ANOVA. $P < 0.05$ was accepted as statistically significant.

Results

RS-504393 Inhibited Murine MCP-1–Induced Chemotaxis

The effects of RS-504393 on murine MCP-1–induced chemotaxis were evaluated. Murine MCP-1 induced spleen cell chemotaxis (Figure 1). In contrast, RS-504393 inhibited the MCP-1–induced migration in a dose-dependent manner, with a 50% inhibitory concentration value of 0.8 μM. Even though murine MIP-1α induced spleen cell chemotaxis in the same way as MCP-1, RS-504393 did not inhibit the MIP-1α–induced migration at all.

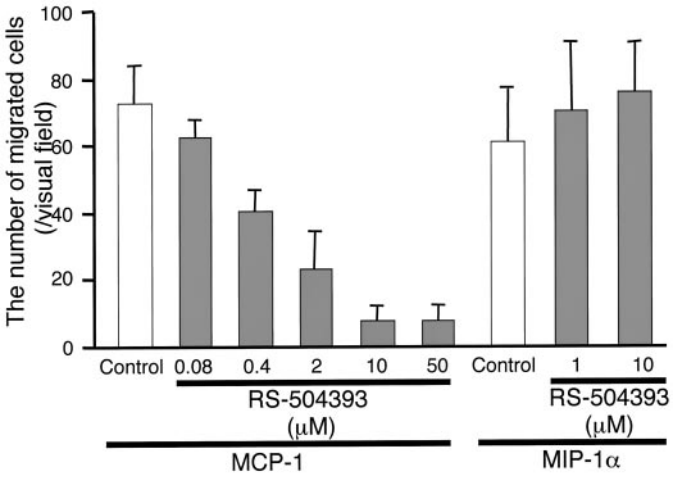

Figure 1. RS-504393 inhibited monocyte chemoattractant protein 1 (MCP-1)–induced chemotaxis. Spleen cells were treated with the indicated concentrations of RS-504393 for 20 min and subjected to MCP-1– or macrophage inflammatory protein 1α (MIP-1α)–induced chemotaxis. The number of migrated cells counted in high-power fields was shown. RS-504393 inhibited MCP-1–induced chemotaxis with a 50% inhibitory concentration value of 0.8 μM. However, RS-504393 did not inhibit MIP-1α–induced chemotaxis. Values are mean ± SEM. $P < 0.05$ *versus* control.

CCR2 Deficiency Reduced the Extent of Acute Tubular Necrosis and Preserved Renal Function

Marked acute tubular necrosis was observed after ischemia-reperfusion in outer medulla in wild-type mice 4, 24, and 48 h after ischemia-reperfusion (Figure 1, A, C, and E). In contrast, tubular necrosis after ischemia-reperfusion in outer medulla was significantly reduced in CCR2-deficient mice at each time point (Figure 2, B, D, and F). The area of acute tubular necrosis in CCR2-deficient mice was significantly lower than that of wild-type mice (Figure 3A). However, acute tubular necrosis was hardly detected in kidneys of sham-operated mice or normal mice (Figures 2G and 3A). Moreover, the number of interstitial infiltrated cells was also decreased in CCR2-deficient mice at each time point (Figure 3B). We did not detect pathologic changes, including acute tubular necrosis or interstitial cell infiltration, both in CCR2-deficient mice (data not shown) or wild-type mice just after 1 hr of ischemia (Figure 2H). Electron microscopical analyses demonstrated that tubular epithelial cells in sham-operated mice have long microvilli (Figure 2I). After 60 min of ischemia, the greater parts of tubular epithelial cells in the outer medulla were necrotic, and the rest of parts of tubular epithelial cells lost their microvilli in wild-type mice (Figure 2J) and CCR2-deficient mice (data not shown) 24 h after ischemia-reperfusion. In contrast, the renal function preserved in CCR2-deficient mice after ischemia-reperfusion injury. The increase of blood urea nitrogen after ischemia-reperfusion was significantly prevented in CCR2-deficient mice compared with wild-type mice 24 h (sham, 21.1 ± 0.7 mg/dl: CCR2 deficient, 18.5 ± 1.4 mg/dl; wild type, 29.8 ± 1.2 mg/dl, $P < 0.001$, $n = 5$, respectively) and 48 h (CCR2 deficient, 24.5 ± 1.2 mg/dl; wild type, 33.6 ±

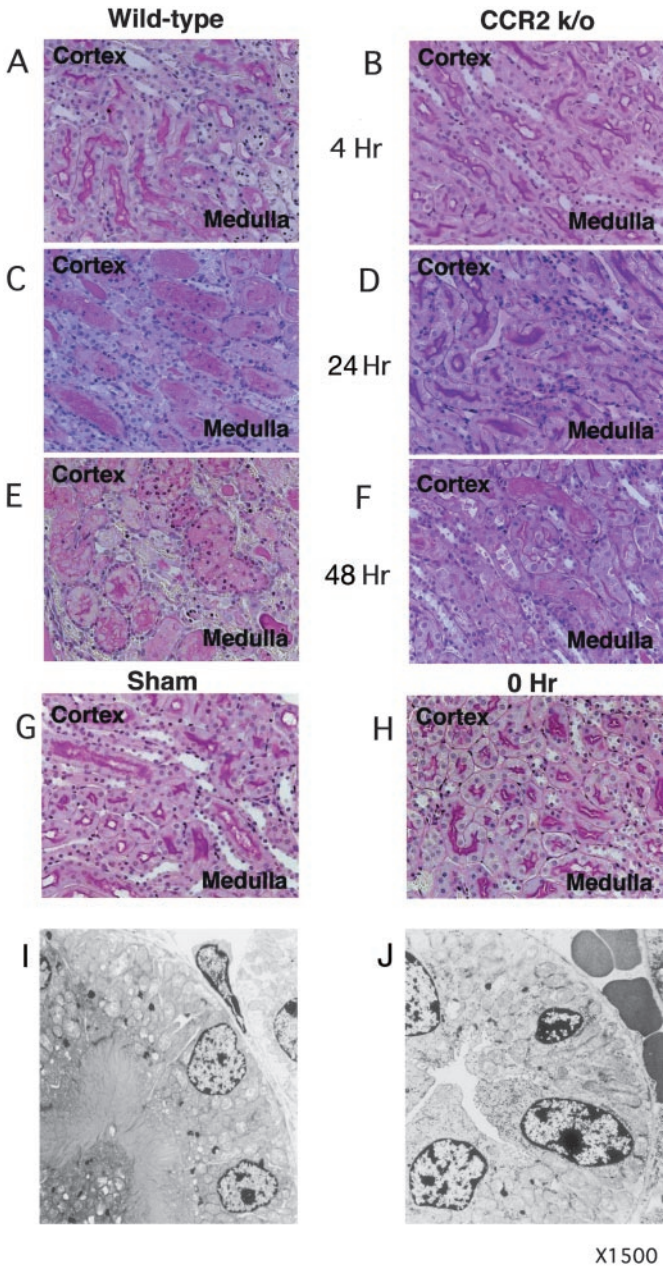

Figure 2. CCR2-deficient mice reduced cell infiltration and acute tubular necrosis in light microscopic and electron microscopic findings. Histopathological examination was performed by periodic acid–Schiff–stained renal tissues. Huge numbers of infiltrated cells and tubular necrosis were mainly observed in outer medulla in left kidney of wild-type mice 4, 24, and 48 h after ischemia-reperfusion (A, C, E). In contrast, the number of infiltrated cells and tubular necrosis decreased in left kidney of CCR2-deficient mice 4, 24, and 48 h after ischemia-reperfusion (B, D, F). (G) Histologic features of sham-operated left kidney. One-hour ischemia did not induce morphologic changes in kidneys of a wild-type mouse (H). Tubular epithelial cells in sham-operated mice have long microvilli (I). After 60 min ischemia, the greater parts of tubular epithelial cells in the outer medulla were necrotic and the rest of parts of tubular epithelial cells lost their microvilli in wild-type mice (J). CCR2 k/o indicates CCR2-deficient mice; wild, wild-type mice. Original magnification, ×200 for light microscopic findings and ×1500 in electron microscopic findings.

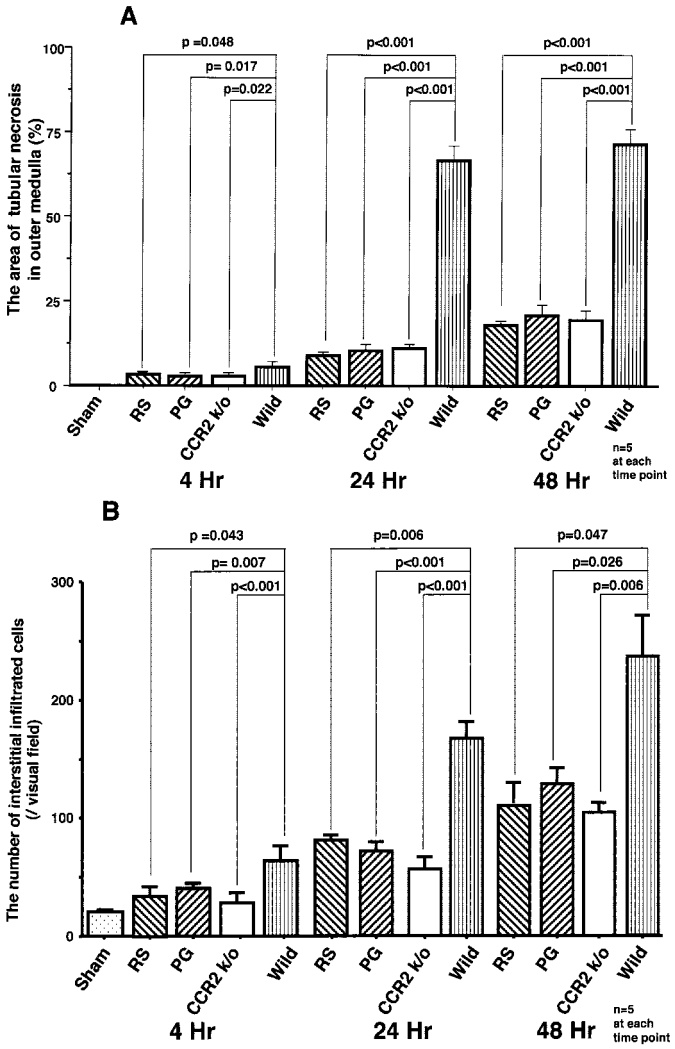

Figure 3. The number of interstitial cells and the area of acute tubular necrosis in outer medulla were reduced in CCR2-deficient mice and propagermanium- or RS-504393–treated mice. Tubular necrosis was evaluated by determining the percentage of tubules in the outer medulla in which epithelial necrosis or necrotic debris was observed. The area of tubular necrosis in CCR2-deficient mice and propagermanium- or RS-504393–treated mice was significantly smaller than that of wild-type mice 4, 24, and 48 h after ischemia-reperfusion in outer medulla (A). The number of interstitial infiltrated cells was counted in randomly selected high-power fields (×400) of outer medulla. The number of interstitial infiltrated cells significantly decreased in CCR2-deficient mice and propagermanium- or RS-504393–treated mice 4, 24, and 48 h after ischemia-reperfusion (B). Each group contained five mice. CCR2 k/o indicates CCR2-deficient mice; PG, propagermanium-treated mice; RS, RS-504393–treated mice; and wild, wild-type mice. Values are expressed as mean ± SEM.

2.6 mg/dl, $P = 0.013$, $n = 5$, respectively) after ischemia-reperfusion.

Interstitial F4/80-Positive Cells Reduced in CCR2-Deficient Mice

Macrophages infiltrated mainly in outer medulla of wild-type mouse kidney 24 and 48 h after ischemia-reperfusion. A significant decrease was observed in the number of interstitial infiltrated F4/80-positive cells in the CCR2-deficient mice 24 and 48 h after ischemia-reperfusion compared with wild-type mice (Figures 2 and 4A). In contrast, there was no significant difference in the number of interstitial infiltrated F4/80-positive macrophages 4 h after ischemia-reperfusion between CCR2-deficient and wild-type mice (Figure 4A). To determine the presence of CCR2 on F4/80-positive cells, a dual-labeled color immunofluorescence immunohistochemistry was used. In wild-type mice, F4/80- (Figure 4B) and CCR2-positive cells (Figure 4D) were detected in ischemia-reperfusion kidney. Most of interstitial infiltrated F4/80-positive cells were also positive for CCR2 in injured kidneys (Figure 4C). CCR2 immunoreactivity was not detected in sections incubated with the absorbed antibodies with excess amount of mouse CCR2 protein (sc-6228P; Santa Cruz Biotechnology) or control goat IgG (Figure 4E), which suggest that this staining was specific to CCR2. In contrast, the cell number in glomeruli did not differ at any time point after ischemia-reperfusion (data not shown).

Interstitial iNOS-Positive Cells Were Reduced in CCR2-Deficient Mice

The iNOS-positive cells were detected immunohistochemically in interstitium of ischemia-reperfusion injured kidneys in wild-type and CCR2-deficient mice (Figure 5A). CCR2-deficient mice decreased the number of interstitial iNOS-positive cells 24 and 48 h after ischemia-reperfusion compared with wild-type mice. In contrast, the number of interstitial iNOS-positive cells did not differ between CCR2-deficient and wild-type mice 4 h after ischemia-reperfusion (Figure 5B).

Reduction of MCP-1 Expression in CCR2-Deficient Mice

To clarify the effect of CCR2 on MCP-1 expression, MCP-1 protein and mRNA in ischemia-reperfusion injury were evaluated. We detected MCP-1 protein in tubular epithelial cells and interstitial infiltrated cells of ischemia-reperfusion injured kidney in wild-type mice 24 h after ischemia-reperfusion (Figure 6A). MCP-1 protein was predominantly expressed in cytosol of tubular epithelial cells. Nevertheless, MCP-1–positive cells were faintly detected in glomeruli of ischemia-reperfusion injured kidney. In CCR2-deficient mice, MCP-1–positive cells were faintly detected in interstitium of ischemia-reperfusion kidneys (Figure 6B). MCP-1 immunoreactivity was not detected in sections incubated with the absorbed antibodies with excess amount of mouse MCP-1 (Figure 6C) or control rabbit IgG (data not shown), which suggests that this staining was specific to MCP-1. Real-time RT-PCR showed that the expression of MCP-1 transcripts in sham-operated left kidneys or contralateral kidneys was low. The expression of MCP-1 significantly increased in diseased kidneys of wild-type mice, which was decreased in CCR2-deficient mice after ischemia-reperfusion (Figure 6D). The correlation of MCP-1 protein and F4/80-positive cells was demonstrated by a dual staining technique in wild-type mice 24 h after ischemia-reperfusion. F4/80-positive cells infiltrated around MCP-1–positive cells

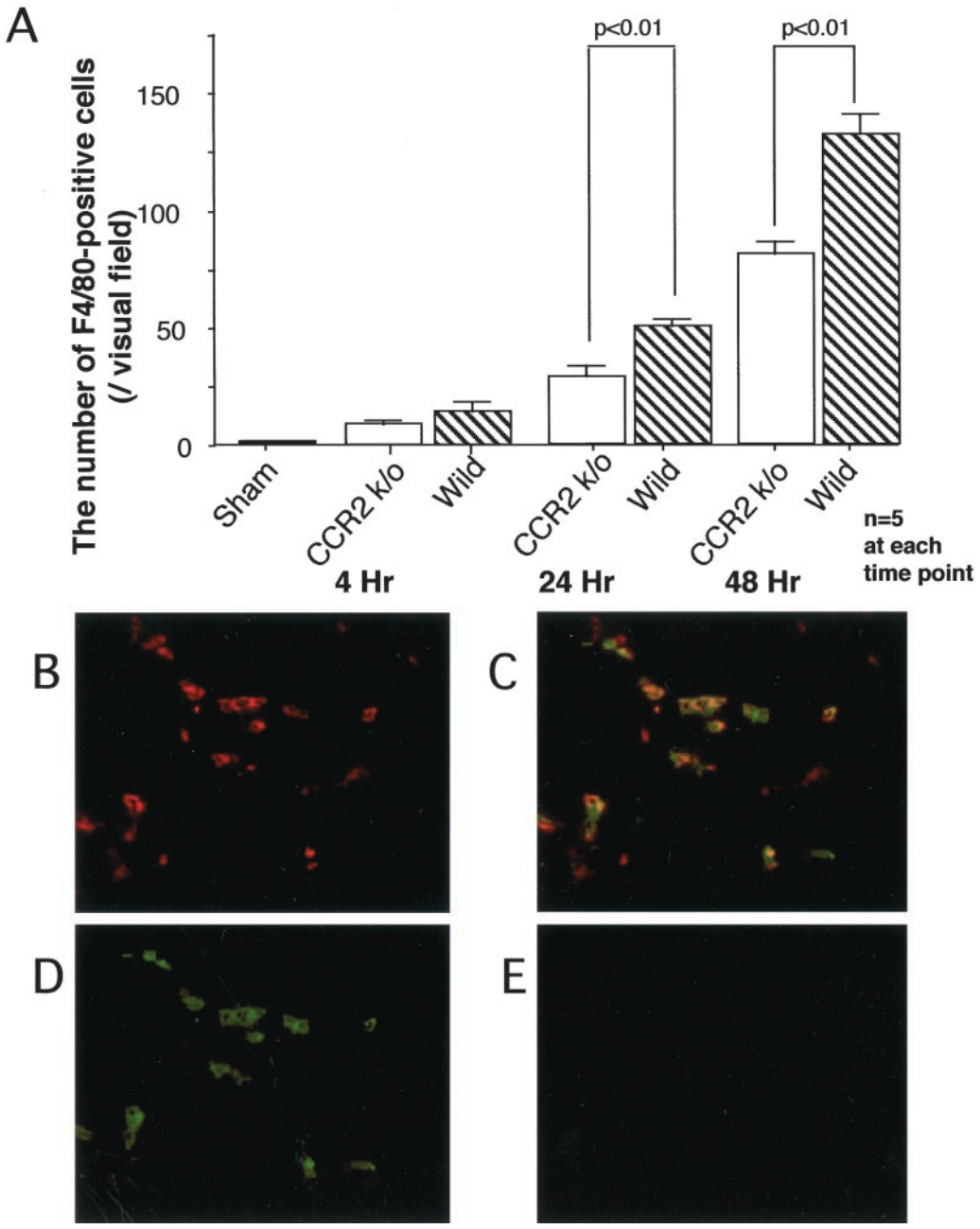

Figure 4. The number of F4/80-positive cells decreased in CCR2-deficient mice. The number of F4/80-positive cells was counted in randomly selected high-power fields (×400) of outer medulla 4, 24, and 48 h after ischemia-reperfusion or sham operation. The number of F4/80-positive cells significantly decreased in CCR2-deficient mice 24 and 48 h after ischemia-reperfusion (A). F4/80-positive cells were visualized with Cy3 and the CCR2 staining with FITC. In wild-type mice, F4/80- (B) and CCR2- (D) positive cells were detected in outer medulla of ischemia-reperfusion kidney 24 h after ischemia-reperfusion. Most of interstitial infiltrated F4/80-positive cells were also positive for CCR2 in injured kidneys (C). CCR2 immunoreactivity was not detected in sections incubated with normal goat IgG (E). Each group contained five mice. CCR2 k/o indicates CCR2-deficient mice; wild, wild-type mice. Values are expressed as mean ± SEM. Original magnification, ×400.

in wild-type mice (Figure 6E). Some interstitial infiltrated F4/80-positive cells were MCP-1 positive (Figure 6E).

Interstitial Granulocytes Were Reduced in CCR2-Deficient Mice

Granulocytes infiltrated mainly in outer medulla of wild-type mouse kidney after ischemia-reperfusion. By use of a monoclonal antibody against granulocytes, a significant decrease was found in the number of interstitial infiltrated granulocytes in the CCR2-deficient mice 4 and 24 h after ischemia-reperfusion compared with wild-type mice (Figures 2 and 7A). However, the number of interstitial infiltrated granulocytes 48 h after ischemia-reperfusion did not differ between CCR2-deficient and wild-type mice (Figure 7A). To determine the

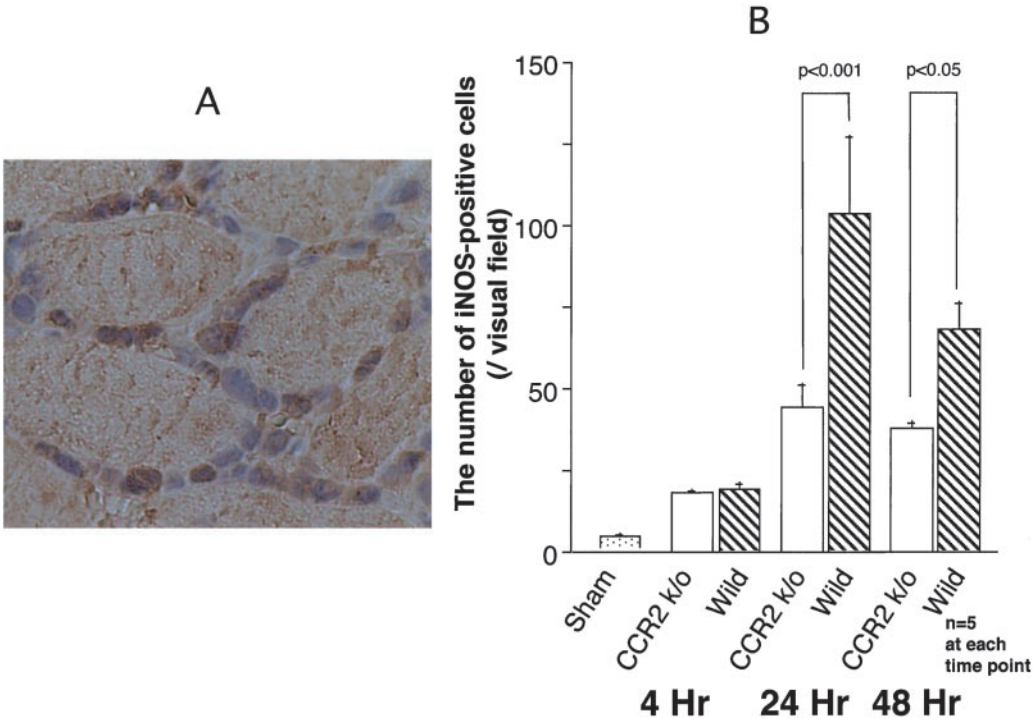

Figure 5. The number of inducible nitric oxide synthetase (iNOS)–positive cells decreased in CCR2-deficient mice. The iNOS-positive cells were detected immunohistochemically in interstitium of ischemia-reperfusion injured kidney for both wild-type mice and CCR2-deficient mice. (A) Wild-type mice 48 h after ischemia-reperfusion. The number of iNOS-positive cells was counted in randomly selected high-power fields (×400) of outer medulla 4, 24, and 48 h after ischemia-reperfusion or sham operation. The number of iNOS-positive cells significantly decreased in CCR2-deficient mice 24 and 48 h after ischemia-reperfusion (B). Each group contained five mice. CCR2 k/o indicates CCR2-deficient mice; wild, wild-type mice. Values are expressed as mean ± SEM. Original magnification, ×400.

presence of CCR2 on granulocytes, we examined a dual-labeled color immunofluorescence immunohistochemistry. In wild-type mice, granulocytes (Figure 7B) and CCR2-positive (Figure 7D) cells were detected in ischemia-reperfusion kidney after reperfusion. A small but significant number of interstitial infiltrated granulocytes was CCR2-positive in injured kidneys (Figure 7C). Ischemia-reperfusion enhanced renal transcripts of KC, MIP-2, and ENA-78, chemotactic for granulocytes, only 4 h after ischemia- reperfusion in wild-type mice. In contrast, ischemia-reperfusion enhanced renal NAP-2 transcripts in wild-type mice 4, 24, and 48 h after ischemia-reperfusion. The enhanced transcripts of KC, MIP-2, ENA-78, and NAP-2 were not detected in CCR2-deficient mice 4, 24, and 48 h after ischemia-reperfusion (Figure 7E). RT-PCR also failed to detect transcripts of KC, MIP-2, ENA-78, and NAP-2 in sham-operated left kidneys (Figure 7E) or contralateral kidneys (data not shown).

Treatment of Propagermanium and RS-504393 Prevented Renal Injury in Wild-Type Mice after Ischemia-Reperfusion

The area of acute tubular necrosis in propagermanium- or RS-504393-treated mice was significantly smaller than that of wild-type mice 4, 24, and 48 h after ischemia-reperfusion (Figure 3A). Furthermore, the area of acute tubular necrosis in propagermanium- or RS-504393–treated mice was the similar level as CCR2-deficient mice 4, 24, and 48 h after ischemia-reperfusion. On the contrary, the area of acute tubular necrosis in outer medulla reduced 96 h after ischemia-reperfusion both in wild-type mice and propagermanium-treated mice, which displayed no difference (propagermanium, 12.2% ± 0.8%; wild type, 13.5% ± 3.4%, NS, $n = 5$, respectively).

Moreover, propagermanium and RS-504393 decreased interstitial infiltrated cells, which were similar to those in CCR2-deficient mice 4, 24, and 48 h after ischemia-reperfusion (Figure 3B). In contrast, interstitial infiltrated cells in outer medulla reduced at 96 h both in wild-type mice and propagermanium-treated mice, and there was no difference between two groups (propagermanium, 68.2 ± 9.5 per visual field; wild-type, 94.4 ± 18.0 per visual field, NS, $n = 5$, respectively). The number of cells in glomeruli did not differ between propagermanium- or RS-504393–treated mice and wild-type mice at any time point after ischemia-reperfusion (data not shown).

Discussion

In this report, we have evaluated the importance of CCR2 signaling in ischemia-reperfusion injury in mice. We now report that acute tubular necrosis and interstitial cell infiltration in ischemia-reperfusion injury were markedly reduced in CCR2-deficient mice and propagermanium- or RS-504393–treated mice compared with wild-type mice up to 96 h after ischemia-reperfusion. We also note that the expression of

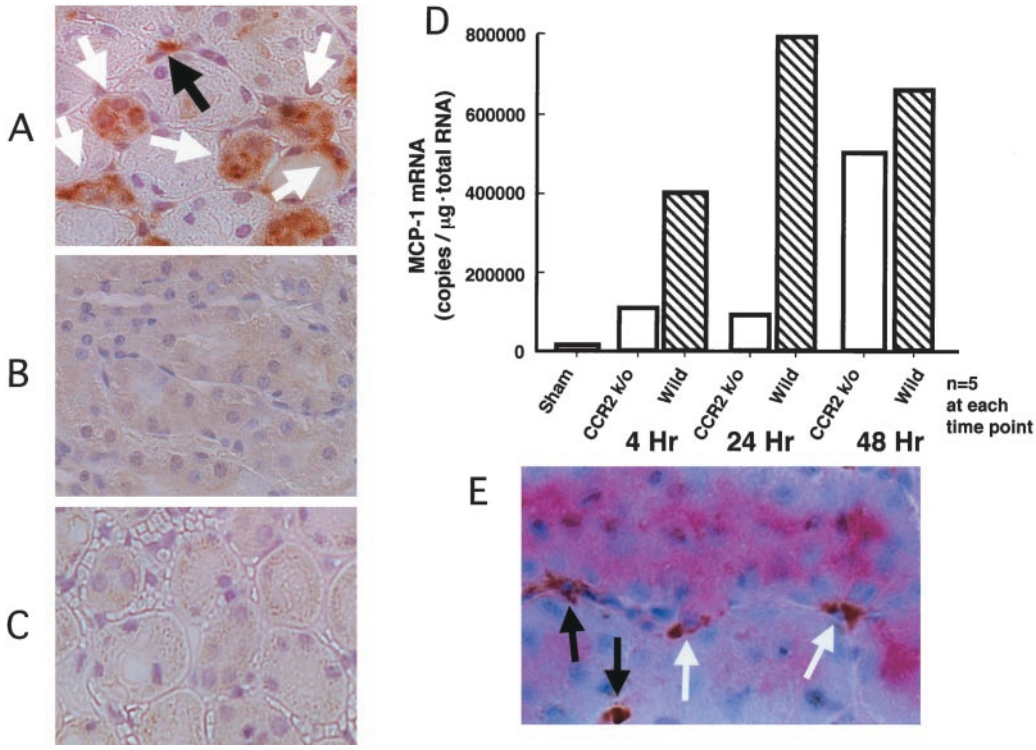

Figure 6. Reduction of monocyte chemoattractant protein 1 (MCP-1) expression in CCR2-deficient mice. The presence of MCP-1 protein was demonstrated immunohistochemically in diseased kidney by the indirect avidin-biotinylated peroxidase complex method. MCP-1–positive cells were detected in tubular epithelial cells (white arrows) and interstitial infiltrated cells (black arrow) in wild-type mice 24 h after ischemia-reperfusion (A). Nevertheless, in CCR2-deficient mice, MCP-1–positive cells were faintly detected in interstitium of ischemia-reperfusion kidneys (B). MCP-1 immunoreactivity was not detected in sections incubated with the absorbed antibodies (C). The expression of MCP-1 mRNA was evaluated with real-time reverse transcriptase–PCR. MCP-1 mRNA expression in each sample was described as copies/μg·total RNA. Ischemia-reperfusion enhanced renal MCP-1 transcripts in diseased kidneys of wild-type mice, which were decreased in CCR2-deficient mice (D). The correlation of MCP-1 protein and F4/80-positive cells was demonstrated by a dual staining technique in wild-type mice 24 h after ischemia-reperfusion. The black arrows indicate F4/80-positive infiltrated cells (brown), and the white arrows indicate dual positive cells for MCP-1 (red) and F4/80 (brown) (E). Each group contained five mice. CCR2 k/o indicates CCR2-deficient mice; wild, wild-type mice. Original magnification, ×400.

MCP-1 and the number of interstitial infiltrated macrophages and iNOS-positive cells were smaller in CCR2-deficient mice than those of wild-type mice. These findings suggest that the presence of CCR2 may be required for macrophage infiltration and subsequent tissue necrosis in renal ischemia-reperfusion injury in mice and that the inhibition of CCR2 signaling by propagermanium or RS-504393 may become a beneficial therapeutic approach against ischemia-reperfusion in kidneys.

In this study, we demonstrate that the expression of MCP-1 in ischemia-reperfusion injury was markedly reduced in CCR2-deficient mice. We report here that MCP-1 was expressed in the injured kidney of wild-type mice after ischemia-reperfusion. Safirstein *et al.* (7) reported that MCP-1 protein was localized to the apical regions of ascending limbs in rat injured kidney of ischemia for 50 min. In this study, we clamped mouse renal vessels for 60 min. Sixty minutes' ischemia was thought to be severe for mouse kidney. As shown in Figure 2, the greater part of tubular epithelial cells in the outer medulla was necrotic, and the cells lost their microvilli and polarity. Moreover, our staining demonstrated that MCP-1 protein was predominantly expressed in cytosol of tubular epithelial cells. Therefore, it is hard for us to determine the segment of tubular epithelial cells and polarity of MCP-1 expression in this experiment. Moreover, we report in this study that the expression of MCP-1 was markedly diminished in CCR2-deficient mice after ischemia-reperfusion. The expression of MCP-1 in tubular epithelial cells was upregulated through TNF-α, which expressed from tubular epithelial cells in hypoxic condition (28–31). MCP-1 promotes macrophage infiltration and activation. In this study, we demonstrated that most of infiltrated macrophages were CCR2 positive. The interstitial activated macrophages produce cytokines and chemokines, which in turn stimulate renal resident cells such as renal tubular epithelial cells or endothelial cells to produce cytokines and chemokines. This augmentative cycle may participate in an increase of MCP-1 production. Moreover, it was recently reported that MCP-1 activates AP-1 and nuclear factor κB in tubular epithelial cells (32), which suggests that deficiency in CCR2 may at least in part be responsible for reduced expression of MCP-1 in interstitium. Therefore, CCR2 deficiency deteriorated this amplification cycle and eventually diminished MCP-1 production.

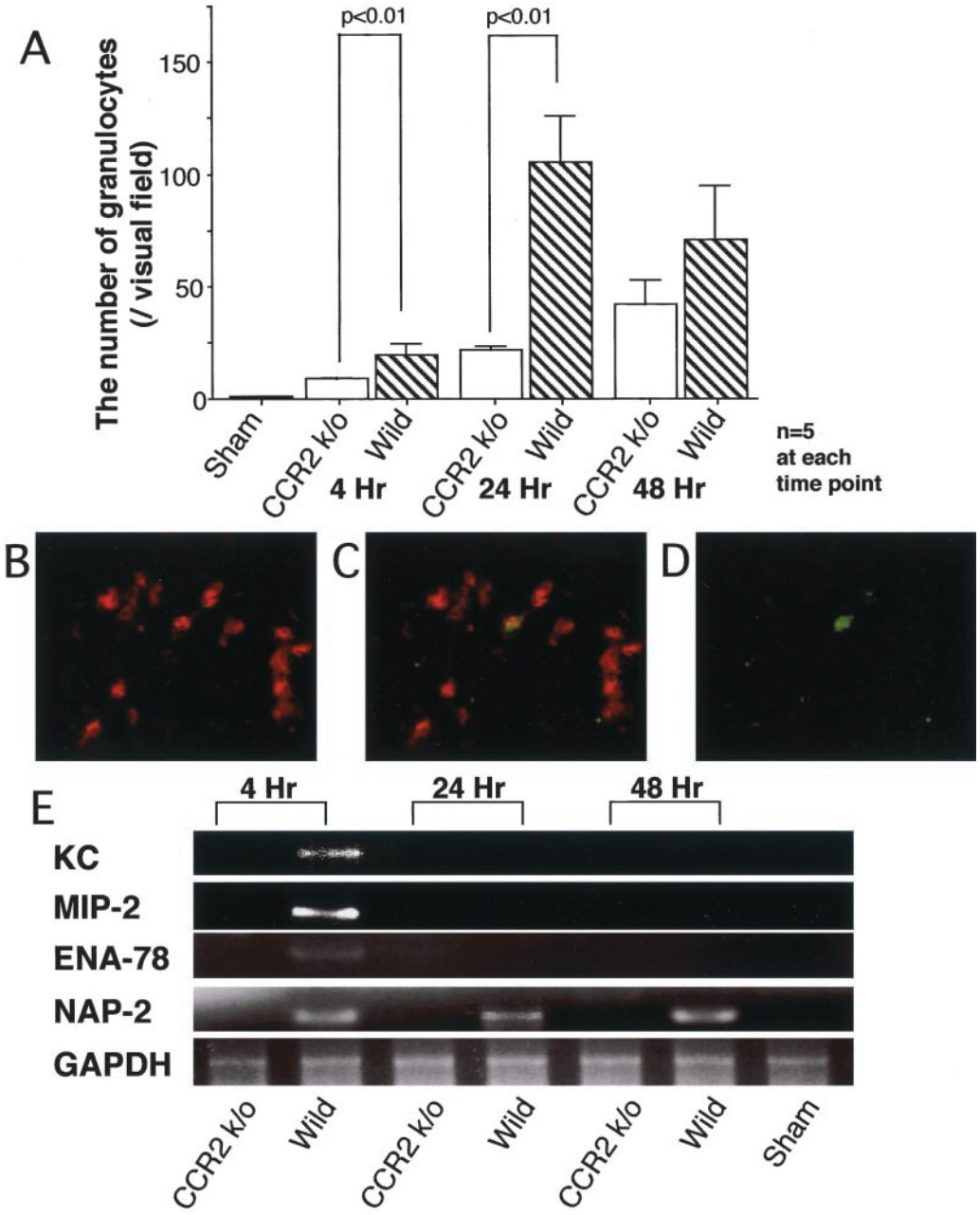

Figure 7. The number of granulocytes decreased in CCR2-deficient mice. The number of granulocytes was counted in randomly selected high-power fields (×400) of outer medulla 4, 24, and 48 h after ischemia-reperfusion or sham operation. The number of granulocytes significantly decreased in CCR2-deficient mice 4 and 24 h after ischemia-reperfusion (A). Granulocyte was visualized with Cy3 and CCR2 with FITC. In wild-type mice, granulocytes (B) and CCR2-positive cells (D) cells were detected in ischemia-reperfusion kidney 4 h after ischemia-reperfusion. A small but significant number of granulocytes was CCR2 positive (C). The expression of KC, macrophage inflammatory protein 2 (MIP-2), neutrophil-activating peptide 78 (ENA-78), and neutrophil-activating peptide 2 (NAP-2) mRNA was evaluated by reverse transcriptase–PCR. KC, MIP-2 and ENA-78 were detected in wild-type mice 4 h after ischemia-reperfusion. NAP-2 was detected in wild-type mice 4, 24, and 48 h after ischemia-reperfusion (E). Each group contained five mice. CCR2 k/o indicates CCR2-deficient mice; wild, wild-type mice. Values are expressed as mean ± SEM.

We demonstrate in this study that the number of interstitial infiltrated macrophages was markedly reduced in CCR2-deficient mice. Alterations in cellular metabolism have been directly related to the severity of ischemic injury in renal tubular epithelial cell structure and function. However, the inflammatory response to ischemia may amplify ischemic injury during the reperfusion period (28,29,33). Nevertheless, most studies on renal ischemia-reperfusion injury have focused on the infiltration and activation of granulocytes (2,3,5). In this study, we focused on the macrophage through the CCR2/MCP-1 signaling. Recently, CCR2-deficient mice, evaluated in several disease models, failed to recruit macrophages (19,34–36).

Macrophages are required for engagement with adhesion molecules to extravasate into the parenchyma of injured kidney. Upregulation of intercellular adhesion molecule 1 and P-selectin have been detected in postischemic kidneys (33,36). Furthermore, inhibition of these adhesion molecules has attenuated cell infiltration in renal ischemia-reperfusion injury (28,37). MCP-1 upregulates the expression of intercellular adhesion molecule 1 in macrophage (38). Therefore, the absence of MCP-1/CCR2 signaling may fail to induce cell infiltration in the diseased kidneys via adhesion molecules, resulting in reduced cell infiltration.

In this study, the reduction in the number of interstitial infiltrated macrophages in CCR2-deficient mice may be proportional to decreased production of MCP-1. The number of interstitial infiltrated macrophages in CCR2-deficient mice, however, was not completely diminished after ischemia-reperfusion. These findings suggest that other chemokines or cytokines than MCP-1, such as MCP-2, -3, or -4, MIP-1α, or RANTES (regulated upon activation, normal T cell expressed and secreted) may participate in macrophage infiltration in ischemia-reperfusion injury. Moreover, we found that the number of iNOS-positive cells was not different between 24 and 48 h after ischemia-reperfusion in CCR2-deficient mice. These findings suggest that MCP-1/CCR2 is more responsible for the activation of inflammatory cells, resulting in the pathogenesis of tubular necrosis. Taken together, the data indicate that CCR2 signaling via MCP-1 plays an important role in renal ischemia-reperfusion injury through the infiltration and activation of macrophages, resulting in the enhanced interaction between renal resident cells and infiltrated cells.

Activation of macrophages is a critical step in macrophage function, resulting in the pathogenesis of tubular necrosis. The precise mechanisms leading to the activation of macrophages in ischemia-reperfusion are insufficiently characterized. We now reveal that CCR2-deficient mice significantly reduced iNOS-positive cells. iNOS may cause the production of sustained high levels of NO. NO is a free radical produced from L-arginine by NOS, and it is involved in diverse processes of inflammation, host defense, vasodilatation, and neurotransmission (39–41). In renal ischemia-reperfusion injury, the contribution of NO is controversial. Some reports revealed that NO might have deleterious effects on ischemia-reperfusion injury; the other reports revealed that NO might have protective effects on ischemia-reperfusion injury (42–44). However, accumulating evidence indicates that the excessive production of NO plays a pathogenic role in both acute and chronic models of inflammation and that NO may be a marker of activated macrophages (45). In addition, MCP-1 upregulates iNOS expression, which participates in tissue damage in various organs (45,46). Furthermore, MCP-1 has been documented to induce release of lysosomal enzymes and generation of superoxide anions, resulting in tissue destruction (47). These data, taken together, imply that MCP-1/CCR2 may be predominantly involved in activation of infiltrated macrophages and may subsequently participate in the pathogenesis of interstitial damage, including tubular necrosis in renal ischemia-reperfusion.

We note that tubular necrosis was also significantly reduced in CCR2-deficient mice 4 h after ischemia-reperfusion. Concomitantly, we found that CCR2-deficient mice reduced the interstitial granulocyte infiltration compared with wild-type mice 4 and 24 h after ischemia-reperfusion. Recent studies revealed that inflammation upregulates CCR2 expression in granulocytes (48). Moreover, several studies reported that MCP-1 and CCR2 promote the chemotaxis of granulocytes during acute (49) and chronic (50,51) inflammatory conditions *in vivo*. Furthermore, we reveal that expression of KC, MIP-2, ENA-78, and NAP-2, which participate in infiltration of granulocytes (52), was upregulated in wild-type mice 4 h after ischemia-reperfusion. Because KC, MIP-2, ENA-78, and NAP-2 are reported to be secreted by monocytes, diminished number of infiltrated monocytes and the blockade of activation of monocytes may reduce the expression of KC, MIP-2, ENA-78, and NAP-2 in CCR2-deficient mice 4 h after ischemia-reperfusion compared with wild-type mice. Moreover, we presume that relationship between infiltrated macrophages and tubular epithelial cells is important for expression of KC or MIP-2. MCP-1 activates AP-1 and NF-κB in tubular epithelial cells (53), and KC and MIP-2 were secreted from tubular epithelial cells through activation of AP-1 and NF-κB (54).

Because the number of interstitial infiltrated macrophages in CCR2-deficient mice tends to diminish, CCR2-deficient mice might have reduction in MCP-1/CCR2-related augmentative signaling cascade and may thereby result in decline in the expression of KC and MIP-2 in tubular epithelial cells. Therefore, taken together, the expression of KC and MIP-2 might be related to the activation of tubular epithelial cells in ischemia-reperfusion in addition to the infiltration and activation of macrophages in diseased kidneys. Furthermore, activated granulocytes are potential source of reactive oxygen species and proteolytic enzymes such as serine-proteases, metalloproteases, thiol-proteases, and aspartate proteases, resulting in tissue destruction (55). Collectively, CCR2 may directly or indirectly participate in granulocyte infiltration, resulting in tubular necrosis in early phase of ischemia-reperfusion injury in mice.

In this study, we used CCR2-deficient and wild-type control animals, which had an outbred C57BL/6J $\times$ 129/Ola genetic background (more than eight generations). The data from these mixed genetic background animals might have a little scattering. We carefully select the same genetic background wild-type animals as a control, and our data were significantly different between CCR2-deficient mice and wild-type mice. To confirm these findings definitely, ischemia-reperfusion injury in CCR2-deficient mice with a single genetic background should be reevaluated in future.

Ischemia-reperfusion injury is responsible for various types of renal injury. For example, all renal allografts suffer unavoidable ischemic injury from the transplantation process. Moreover, the extent of acute tubular necrosis at the transplantation may influence the long-term outcomes of transplanted kidneys (56). Furthermore, early granulocyte-mediated damage after ischemia-reperfusion influences the renal function of transplanted graft (57). Thus, prevention of acute renal injury at the transplantation may be ideal for the better outcomes of renal

transplantation. This study demonstrated that the inhibition of CCR2 reduced ischemia-reperfusion injury in kidneys. Therefore, postnatal regulation of CCR2 signaling is useful therapeutic approach for ischemia-reperfusion injury. A selective inhibitor against CCR2 has not been clinically available. Propagermanium binds to the N-terminal peptides of CCR2 together with glycosylphosphatidylinositol-anchored protein and inhibits CCR2 function (20). Moreover, propagermanium does not affect the function of other chemokines, such as IL-8, RANTES and MCP-1, and CCR2 expression (20). This drug has been used as a therapeutic agent against hepatitis B virus–induced hepatitis in Japan. Therefore, regulation of MCP-1/CCR2 signaling possibly by this drug may be a safe and effective therapeutic tool for ischemia-reperfusion injury in kidney.

In addition to propagermanium, we tried to examine the effects of another CCR2-specific antagonist, RS-504393. RS-504393 inhibited MCP-1–induced chemotaxis in a dose dependent manner. On the contrary, RS-504393 did not inhibit MIP-1α–induced chemotaxis. In addition to our data, the specific affinity to chemokine receptors and chemotaxis inhibition against CC chemokines were evaluated on THP-1 cells (21). Taken together, these data revealed that RS-504393 actually works as the MCP-1–specific antagonist in mice. Moreover, RS-504393 prevented interstitial cell infiltration and tubular necrosis, which was similar to those of CCR2-deficient mice and propagermanium-treated mice 24 and 48 h after reperfusion. Even though propagermanium might have other effects than inhibition of CCR2 function, the main effects of propagermanium in ischemia-reperfusion injury in kidney is presumed to depend on CCR2 inhibition. Taken together, these results indicate that the inhibitory effects of propagermanium or RS-504393 further strengthen the therapeutic potential by CCR2 inhibition for ischemia-reperfusion injury.

In summary, we found that the inhibition of CCR2 markedly ameliorated inflammatory cell infiltration, resulting in reduced tubular necrosis in renal ischemia-reperfusion. These results suggest that CCR2 and its cognate ligand, MCP-1, play important roles in the pathogenesis of renal ischemia-reperfusion injury and offer therapeutic targets for ischemia-reperfusion in kidneys.

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