Gene Therapy Expressing Amino-Terminal Truncated Monocyte Chemoattractant Protein-1 Prevents Renal Ischemia-Reperfusion Injury

KENGO FURUICHI,* TAKASHI WADA,* YASUNORI IWATA,* KIYOKI KITAGAWA,* KEN-ICHI KOBAYASHI,* HIROYUKI HASHIMOTO,# YOSHIRO ISHIWATA,† NAOHISA TOMOSUGI,‡ NAOFUMI MUKAIDA,¶ KOUJI MATSUSHIMA,‡ KENSUKE EGASHIRA,§ and HITOSHI YOKOYAMA*

*Department of Gastroenterology and Nephrology, Graduate School of Medical Science, and Division of Blood Purification, ¶ Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan; # Sanwa Kagaku Kenkyusho Co., Ltd., Inabe, Japan; † Division of Internal Medicine, Kanazawa Medical University, Uchinada, Japan; ¤ Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and § Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Abstract. Ischemia-reperfusion is closely associated with tissue damage in various organs, including kidney. Despite clinical investigations, useful therapy for renal ischemia-reperfusion injury is not available so far. This study evaluated therapeutic effects of gene therapy expressing an amino-terminal deletion mutant of MCP-1 called 7ND to inhibit monocyte chemoattractant protein (MCP)-1/CCR2 signaling in vivo on renal ischemia-reperfusion injury. 7ND gene was transferred into the femoral muscle of Balb/c mice. Renal artery and vein of the left kidney were occluded with a vascular clamp for 60 min. A large number of infiltrated cells were observed, as was marked acute tubular necrosis in outer medulla after renal ischemia-reperfusion injury in control mice, while these lesions were significantly decreased in 7ND gene-transfected mice. Macrophages in the interstitial region, most of which were CCR2-positive, were markedly decreased in 7ND gene-transfected mice after reperfusion. Although macrophages infiltrated around MCP-1-positive cells in control mice, the smaller number of F4/80-positive cells could infiltrate into the neighbor of MCP-1-positive cells in 7ND-treated mice. These results provide evidence that gene therapy by 7ND is potentially a powerful therapeutic approach to inhibit MCP-1/CCR2 signaling, resulting in rescue from renal ischemia-reperfusion injury.

Monocyte chemoattractant protein (MCP)-1 (also termed as monocyte chemotactic and activating factor), a well-known member of CC chemokine family, plays key roles in infiltration and activation of macrophages and results in tissue destruction, including renal diseases (3–5). Conversely, recent studies revealed that CCR2 signaling is also involved in tissue destruction of renal diseases (5–8). We therefore hypothesized that such blockade of MCP-1/CCR2 signaling pathway in vivo could suppress MCP-1/CCR2-mediated inflammation and thereby improve the destruction of renal ischemia-reperfusion injury. To achieve this goal, we evaluated gene therapy with electroporation to block MCP-1/CCR2 signaling pathway in vivo using an amino-terminal truncated MCP-1 called 7ND, which has been shown to bind to CCR2 and subsequently block MCP-1-mediated monocyte chemotaxis in vitro (9). The novel findings of this study are that postnatal blockade of MCP-1/CCR2 signaling pathway using 7ND markedly prevented after renal ischemia-reperfusion. We thus suggest that MCP-1/CCR2 signaling may be the possible therapeutic target for ischemia-reperfusion injury.
Materials and Methods

Expression of 7ND

Human 7ND cDNA with an epitope tag FLAG (DYKDDDDK) in the carboxyl-terminal was constructed by recombinant PCR using a wild-type human MCP-1 cDNA (a gift of Dr. Tezio Yoshimura, NCI, Frederick, MD) as template and cloned into the BamHI(5') and NddI(3') sites of the pcDNA3 expression vector plasmid (Invitrogen, Carlsbad, CA). The expression of 7ND with FLAG in skeletal muscle was detected by immunohistochemical method using anti-FLAG M2 antibody (Sigma-Aldrich, Munich, Germany) 4 h after reperfusion. FLAG-conjugated 7ND in the serum was detected using FLAG Western detection kit (Stratagene, La Jolla, CA). In brief, to determine 7ND in the serum, SDS-polyacrylamide gel electrophoresis was carried out using SDS-page gels and SDS running buffer, and then the protein bands from the gels were electroblotted onto nitrocellulose membranes. FLAG Western detection kit was used for detecting immunoreactivity. Moreover, we evaluated the serum levels of 7ND using a quantitative sandwich enzyme immunoassay for human MCP-1 (R&D Systems, Minneapolis, MN). This assay does not crosreact other chemokines, including murine MCP-1.

Animals and Transfection of 7ND Gene

Inbred male Balb/c mice, aged 8 wk, were obtained from Charles River, Japan Inc. (Atsugi, Kanagawa, Japan). Three groups of Balb/c mice were studied: the sham-operated group; the control group, which received an administration of 50 µl of empty plasmid (1 µg/µl); and the 7ND gene-transfected group, which received an administration of 50 µl of plasmid (1 µg/µl) containing the 7ND gene into the femoral muscle. To enhance expression, the animal received electroporation using an electric pulse generator ECM830 (BTX, San Diego, CA) at the injected site immediately after injection.

All procedures employed in the animal experiments complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Renal Ischemic Model

Seven days after gene delivery, the renal artery and vein of the left kidney were occluded for 60 min. Zero, 4, 24, or 48 h after reperfusion, renal tissues from five mice at each time point were removed 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 (PAS) reagent. Two independent observers with no prior knowledge of the experimental design evaluated each tissue section. The number of interstitial infiltrated cells was counted in 20 randomly selected high-power fields (×400) of outer medulla. The observers scored with a previously described semiquantitative scale designed to evaluate the degree of tubular necrosis (10). Higher score represented severe damage (maximum score, 4): 0, normal kidney; 1, <5% involvement; 2, 5 to 25% involvement; 3, 25 to 75% involvement; 4, >75% involvement.

Immunohistochemical Studies

The other portion of fresh renal tissue of 24 h after reperfusion, embedded in OCT compound and snap-frozen, was cut at 6 µm. The presence of F4/80-positive macrophages, CCR2-positive cells, and MCP-1-positive cells was detected immunohistochemically by using rat anti-mouse F4/80 monoclonal antibody (clone: A3–1; BMA Bio-

Detection of MCP-1 Transcripts in Diseased Kidneys

To determine the MCP-1 transcripts, total RNA was extracted from the whole kidneys from five mice in each group 4 h after reperfusion. cDNA was reverse-transcribed from 5 µg of total RNA, combined from five mice in each group (1 µg of RNA per a mouse), by using a SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was performed using 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 PCR was performed in the LightCycler using the LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics). Primers for MCP-1 (forward: 5'-ACTGAAGCCAGCTCTCTCTGTCC-3', reverse: 5'-TTCCTTCTTGGGTCACAGACAG-3') (11) and GAPDH (Nihon Gene Research Lab’s, Inc., Sendai, Japan), and 2 µl of the cDNA prepared above were used to detect MCP-1 and GAPDH. 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 after correction with GAPDH expression. The expression of MCP-1 and GAPDH in each sample was quantified in separate wells. No PCR products were detected in the real-time PCR procedure without reverse transcription, indicating that the contamination of genomic DNA was negligible. Gels of the PCR products after quantification of MCP-1 or GAPDH by real-time PCR showed a single band (270 and 230 bp, respectively) with the expected size (data not shown). Moreover, we evaluated the serum levels of murine MCP-1 using a quantitative sandwich enzyme immunoassay for murine MCP-1 (R&D Systems). This assay dose not crosreact other chemokines.

Statistical Analyses

The mean and SEM were calculated on all the parameters determined in this study. Statistical analyses were performed using
Kruskal-Wallis test and ANOVA test. $P < 0.05$ was accepted as statistically significant.

Results

MCP-1 Expression 4 h after Ischemia-Reperfusion

We detected MCP-1-positive cells among tubular epithelial cells and interstitial infiltrated cells equivalently in the kidney of control mice and 7ND gene-transfected mice 4 h after reperfusion (data not shown). Moreover, the expression of MCP-1 mRNA in injured kidney was increased in both control mice and 7ND gene-transfected mice compared with sham-operated mice (MCP-1/GAPDH: sham, 92.3; wild, 315.6; 7ND, 758.3). Furthermore, serum levels of MCP-1 in diseased kidneys did not differ between control mice and 7ND gene-transfected mice 4 h after reperfusion (sham, 57.0 ± 21.0 pg/ml; wild, 201.0 ± 41.7 pg/ml; 7ND, 198.0 ± 12.3 pg/ml; $n = 5$, respectively). These results suggested that ischemia-reperfusion itself induced renal damage equally in control mice and 7ND gene-transfected mice 4 h after reperfusion.

Expression of 7ND

The number of fibers positive for 7ND was observed in the femoral muscle from the 7ND gene-transfected mice 4 h after reperfusion (Figure 1A). In contrast, a carboxyl-terminal FLAG epitope-tagged 7ND gene was not detected in the muscles from the control group (Figure 1B). Moreover, Western blot analysis of the serum for FLAG protein showed that FLAG epitope-tagged 7ND was efficiently secreted into the serum in 7ND gene-transfected mice (Figure 1C). Furthermore, serum levels of 7ND increased during the experiments (sham, not detectable; 4 h, 47.4 ± 4.3 pg/ml; 24 h, 36.0 ± 6.5 pg/ml; 48 h, 35.2 ± 4.0 pg/ml; $n = 5$, respectively).

7ND Treatment Significantly Decreased the Extent of Acute Tubular Necrosis and Interstitial Cell Infiltration

Marked acute tubular necrosis and a large number of cell infiltrations were induced after ischemia-reperfusion in outer medulla in control mice 4, 24, and 48 h after ischemia-reperfusion in control mice (Figure 2, A, B, F, and G). The scores of acute tubular necrosis in 7ND gene-transfected mice were significantly lower than in those of control mice 24 and 48 h after reperfusion in outer medulla (Figure 2F). The total number of interstitial infiltrated cells significantly decreased in 7ND gene-transfected mice 24 and 48 h after reperfusion (Figure 2G). 7ND indicates 7ND gene-transfected mice.
significantly lower in control mice 24 and 48 h after ischemia-reperfusion (Figure 2, C, D, and F). Moreover, 7ND treatment decreased the number of infiltrated cells 4, 24, and 48 h after ischemia-reperfusion compared with control mice (Figure 2, C, D, and G). The histologic features of sham-operated left kidneys and right control kidneys were essentially same as normal kidneys (Figure 2E).

**Interstitial F4/80-Positive Cells Reduced in 7ND Gene-Transfected Mice**

We observed a significant decrease in the number of interstitial infiltrated F4/80-positive cells in 7ND gene-transfected mice 24 and 48 h after reperfusion compared with control mice (Figure 3A). F4/80-positive (Figure 3B) and CCR2-positive cells (Figure 3D) were detected in ischemia-reperfusion kidney 24 h after reperfusion. Most of interstitial infiltrated F4/80-positive cells were also positive for CCR2 in injured kidneys 24 h after reperfusion (Figure 3C). F4/80-positive cells infiltrated around MCP-1-positive cells in control mice 24 h after reperfusion (Figure 3D). However, the smaller number of F4/80-positive cells infiltrated around MCP-1-positive cell in 7ND gene-transfected mice 24 h after reperfusion (Figure 3F).

**Discussion**

In this report, we have demonstrated that postnatal blockade of MCP-1/CCR2 signaling pathway by the intramuscular transfer of a mutant gene, 7ND, significantly reduced renal injury in ischemia-reperfusion, including acute tubular necrosis and interstitial cell infiltration. Concomitantly, macrophages, most of which were CCR2-positive, were greatly reduced by 7ND. Taken together, inhibition of MCP-1/CCR2 signaling pathway with 7ND gene is the profitable therapy for ischemia-reperfusion injury in kidney.

This study strongly indicates MCP-1/CCR2 signaling pathway as the therapeutic target in renal ischemia-reperfusion injury. We have now demonstrated that the number of interstitial infiltrated macrophages was markedly reduced in 7ND gene-transfected mice. MCP-1 is one of the most vigorous chemoattractant factors for macrophages, and CCR2 is the prominent cognate receptor for MCP-1. We demonstrated that most of infiltrated macrophages in injured kidney were CCR2-positive. In addition to chemotaxis, MCP-1 also engages the expression of adhesion molecules, resulting in extravasation of macrophages into the parenchyma of injured kidney (18).

Furthermore, MCP-1 has been documented to induce release of lysozomal enzymes and generation of superoxide anions from macrophages, resulting in tissue destruction (19). Concomitantly, CCR2-deficient mice failed to recruit macrophages in several disease models (7,8,20). These data suggested the critical contribution of CCR2 in macrophage activation as well as recruitment into diseased organs, leading to tissue damage. Taken all together, MCP-1/CCR2 signaling pathway is important for ischemia-reperfusion injury, and its inhibition may be effective to quench renal injury possibly via diminution of macrophage infiltration and activation.

This study revealed the therapeutic potency of 7ND gene transfer in ischemia-reperfusion injury. It was recently reported that amino-terminal truncated chemokines, such as regulated upon activation, normal T cell expression and secreted (RANTES) and MCP-3, still bind their respective receptors without inducing the biologic response, and are thus true competitive receptor antagonists (21). Moreover, amino-terminus of MCP-1 is essential for MCP-1 to keep chemoattractant activity (22). Furthermore, it was reported that 7ND forms inactive heterodimer formation with wild-type MCP-1 and inhibits monocyte chemotaxis in vitro (9,21). On the basis of these, 7ND gene transfer protects the formation of arteriosclerosis and coronary vascular remodeling thus far (23,24). Therefore, 7ND gene transfer may effectively block MCP-1/CCR2 signaling pathway and prevent progression of MCP-1/CCR2-related diseases. No apparent side effects were observed during period of this study; however, careful observation over a long period of time will be needed in future studies.

This study revealed that the number of neutrophils was reduced in 7ND-treated mice. We and other researchers previously reported the importance of neutrophils in ischemia-reper-

![Figure 3](image-url)
fusion injury (25,26). A recent study revealed that inflammation upregulates CCR2 expression in neutrophils (12). Moreover, several studies reported that MCP-1 and CCR2 promote the chemotaxis of neutrophils during acute (13) and chronic (14,15) inflammatory conditions in vivo. Because CXC chemokines, which participate in infiltration of neutrophils (16) are reported to be secreted by monocytes, diminished the number of infiltrated monocytes and the blockade of activation of monocytes with 7ND may reduce CXC chemokine expression. Furthermore, activated neutrophils are a potential source of reactive oxygen species and proteolytic enzymes such as serine-proteases, metalloproteases, thiol-proteases, and aspartate proteases, resulting in tissue destruction (17). Collectively, 7ND gene transfer may participate in neutrophil infiltration, resulting in tubular necrosis in early phase of ischemia-reperfusion injury in mice.

Even though so many experiments and arguments on anti-MCP-1 therapy against inflammation have been discussed, specific and effective therapy against ischemia-reperfusion injury is not available so far. MCP-1 has been proved to be involved not only in ischemia-reperfusion injury but also in various types of inflammation using MCP-1 itself or CCR2-deficient mice (7,8,13,27). Therefore, regulation of MCP-1/CCR2 signaling leading to macrophage infiltration and activation may have therapeutic potential in inflammation, including renal injury. Thus, 7ND will be an appealing therapeutic approach of great efficacy and efficiency to ischemia-reperfusion injury in kidneys.

Acknowledgments

We thank Dr. Mariko Akiyama (Kanazawa University), Dr. Hui Wang (Kanazawa University), Dr. Toshikazu Kondo (Kanazawa University), and Dr. Chu Kataoka (Kyushu University) for excellent technical advice. TW is a recipient of a Grant-in-Aid (No. 14571019) from the Ministry of Education, Science, Sports and Culture of Japan.

References


Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.


