A free radical scavenger but not FGF-2-mediated angiogenic therapy rescues myonephropathic metabolic syndrome in severe hindlimb ischemia

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IN THE LAST DECADE, a number of experimental studies have suggested the possible utility of angiogenic growth factors (“therapeutic angiogenesis”) to treat limb ischemia, as well as ischemic heart diseases (30, 31, 36). Emerging evidence in clinical trials for limb ischemia, however, has shown a relatively limited outcome of therapeutic angiogenesis, in both protein and gene therapies, in double-blinded placebo-controlled studies (17, 23, 26). Therefore, further effort should be put into the clinical evaluation of the potentials of therapeutic angiogenesis, including the choice of the angiogenic factors, optimized dose and local level of the dose, and indications for clinical stages (4, 5).

In a recent series of experimental studies, we demonstrated that intramuscularly boosted expression of basic fibroblast growth factor (bFGF or FGF-2), which is a prototype polypeptide for angiogenesis, by a highly efficient gene transfer vector, recombinant Sendai virus (SeV) (37), constantly showed efficient therapeutic effects in acute severe hindlimb ischemia of mice (19, 21, 25, 32), as well as chronic limb ischemia of rabbits (25). These data revealed the critical role of endogenous angiogenic factors that are induced by FGF-2, including vascular endothelial growth factor (VEGF) (19, 32) and hepatocyte growth factor (HGF) (21), and more importantly, the data revealed that the effect of FGF-2 was highly dose dependent, requiring >2.5-fold higher local protein level compared with the endogenous expression of it in mice (25).

The indication of angiogenic therapy may possibly be extended from no-choice patients with critical limb ischemia to the advaut therapy for individuals with arterial reconstruction, when its efficacy in the clinical setting can be determined. As part of this extension, myonephropathic metabolic syndrome (MNMS) (7) should be an important target of this new therapeutic strategy. MNMS, a lethal disease after 6 h of “golden time” of vascular reconstruction for acute arterial obstruction, is characterized by pentalogy (oliguria, hyperkalemia, metabolic acidosis, myoglobinemia, and azotemia), affecting multiple organ failure, and results in the death of patients at a high rate (7). Because MNMS is a fatal state of ischemia-reperfusion (I/R) injury of the lower extremity (2, 7), boosted expression of FGF-2 may have a beneficial effect because several experimental studies demonstrated the protective property of FGF-2 against I/R injury in the heart (12, 13, 22, 24). Importantly, FGF-2 is known to have a direct protective activity for skeletal muscles undergoing ischemia-induced injury (18), a finding supported by our previous study using a mouse model of acute severe hindlimb ischemia (19).

For these reasons, we here examined whether SeV-mediated FGF-2 gene transfer might rescue the rat model of MNMS, directly compared with the effect of a newly developed and now clinically available drug of a free radical scavenger, MCI-186 (edaravone), which has been shown to attenuate I/R injury in several organs (10, 29). Unexpectedly, we here show that only MCI-186, but not FGF-2, rescued MNMS in the rat model.
MATERIALS AND METHODS

Animals. Six-week-old male Wistar rats, weighing 200–250 g (KBT Oriental, Charles River grade, Tosu, Saga, Japan), were used in this study. The experimental protocol using animals was submitted to and approved by the Institutional Committees for Animal Experiments and Experiments for Recombinant DNA and Infectious Pathogens at Kyushu University (approval No. 11-76 and 16-94). The animal experiments were done in accordance with recommendations for the proper care and use of laboratory animals, and the law (No. 105) and notification (No. 6) of the Japanese government were also followed.

Gene transfer agents and MCI-186. High titer stocks of SeVs expressing human FGF-2 (SeV-hFGF2) and firefly luciferase (SeV-luciferase) used in this study were prepared as described previously (19, 21, 32, 37). Virus titer was determined by hemagglutination assay using chicken red blood cells and was kept at 80°C until use. MCI-186 was a kind gift from Mitsubishi Pharma.

Experimental protocol. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The surgical procedure for I/R was performed as described in Fig. 1A. Briefly, ischemia was induced by occluding the abdominal aorta under the renal artery and bilateral femoral arteries by 5-0 nylon for 4 h. Reperfusion was then done by removing the nylon threads. The experimental groups were designed as shown in Fig. 1B. For group 3, 0.5 ml of vector solutions, containing 1 × 10^9 plaque-forming units of SeV-hFGF2, was injected into five portions of each lower limb (3 for the thigh and 2 for the calf) intramuscularly 48 h before surgery-induced ischemia. For group 4, 0.5 ml of MCI-186 solution (10 mg/kg in PBS) was administered intravenously 5 min before induced ischemia and intraperitoneally 5 min before reperfusion. For group 2, as a control of group 4, the same volume of PBS was administered instead of MCI-186. Six hours after reperfusion, all animals were killed by an overdose of anesthetic, and materials were subjected to the following evaluations. Group 1 consisted of untreated animals. Three independent experiments (tracks 1–3) were done to obtain different materials from rats, as indicated in Fig. 1C, including two animals that received SeV-luciferase as a control for group 3 in tracks 1 and 2, showing similar results to those seen in group 2 (data not shown). Therefore, a total of 68 animals were used in this study, and no animal died in the series of experiments.

Biochemical analysis. Serum levels of blood urea nitrogen (BUN), creatinine (Cr), potassium (K), creatine phosphokinase (CPK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and aldo...
lase were measured in serum samples taken from the abdominal aorta when all animals were killed at track 2. Concentrations of BUN, Cr, K, CPK, AST, LDH, and aldolase were determined by an automatic analyzer (Research Testing Department, SRL, Hachioji-shi, Tokyo).

Wet-to-dry weight ratio. The left gastrocnemius muscle was excised from animals at track 2, and the wet weight was determined. The muscle was dried at 60°C in a convection oven for 72 h, and then the dry weight was measured. The wet-to-dry weight ratio was calculated as an indicator of muscle edema (11).

Immunohistochemistry. Infiltrated neutrophils within lower limb muscle and lung were identified by immunohistochemistry. Right gastrocnemius muscle (at 10 mm below the knee) and right lung were excised from animals at track 2 and fixed in 10% buffered formalin. Each section was embedded in paraffin, and 2-μm-thick sections were stained with hematoxylin and eosin; additionally, the serial sections were subjected to immunohistochemical studies. Rabbit anti-myeloperoxidase (anti-MPO) (Dako, Glostrup, Denmark, 1:200 in PBS) (1, 38) was used for a primary antibody. Immunohistochemical examinations were performed as described previously (20). In brief, deparaffinized sections were incubated with 3% nonfat milk, and the reaction with peroxidase-labeled secondary antibody (Envision System, Dako) followed reaction of the primary antibody. Positive reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride to give the reaction product a brown color, and then the sections were counterstained with hematoxylin. Nonimmune rabbit IgG was also used instead of the respective primary antibody as each negative control. MPO-positive cells in randomly selected 50 foci of high-powered view (×200) per slide were counted under a light microscope, and the mean value was used as the MPO-positive cells/focus in each section.

ELISA. Serum and/or muscular levels of FGF-2, growth-related oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) (a rodent homolog of human IL-8) (33, 34), and soluble intercellular adhesion molecule-1 (sICAM-1) were determined by ELISA. Commercially available assay systems were used [R&D Systems (Minneapolis, MN) for FGF-2 and sICAM-1, and IBL (Gunma, Japan) for GRO/CINC-1], according to the manufacturers’ instructions. Quantikine system for FGF-2 is available for human, murine, rabbit, rat, and monkey FGF-2 (19, 21, 25, 32). For muscle preparation, the right gastrocnemius muscle was homogenized in lysis buffer with protease inhibitor cocktail (19, 21, 25, 32), the insoluble material was removed by centrifugation at 12,000 rpm for 10 min, and the supernatant was subjected to ELISA. Serum samples were used for ELISA after appropriate dilutions. All samples were stored at −20°C and analyzed within 24 h after collection.

White blood cell count and white blood cell differential count for neutrophils. The white blood cell (WBC) count was determined with an automatic hemocytometer using whole blood taken from the abdominal aorta when all animals were killed at track 3. The neutrophil count was determined from a smear of each sample with Giems staining.

Statistical analysis. Data were presented as means ± SE. All statistical comparisons were performed using one-way ANOVA with Fisher protected least significant difference test for multiple comparisons. A probability value of P < 0.05 was considered significant.

RESULTS

Local concentration of FGF-2. Using muscular tissue at track 1, we determined the local level of FGF-2 to investigate the possible alteration of FGF-2 expression due to the intervention and the level of SeV-hFGF2 gene transfer.

As shown in Fig. 2A, all animals except for those in group 3 (FGF-2) showed no significant increase of local FGF-2 level, indicating that the surgical treatment for I/R did not affect FGF-2 expression. The protein level of FGF-2 in group 3 reached >2.5-fold greater than that seen in untreated animals (group 1), a level that showed a significant hindlimb-salving effect of FGF-2 gene transfer in a murine severe limb ischemia model, as previously demonstrated (25).

The free radical scavenger MCI-186, but not FGF-2, inhibits muscular and renal damage. Before the start of the main experiments shown in Fig. 1, B and C, we determined the effective dose and the timing of MCI-186 administration in use of relatively small numbers of animals (n = 3/each group) by assessing serum CPK, AST, LDH, and aldolase as an initial study (Fig. 2B). We here used two independent doses (3 vs. 10 mg/kg) with dual injection and 10 mg/kg at single (ip 5 min before reperfusion) vs. dual (iv 5 min before induced ischemia and ip 5 min before reperfusion) injection. As a result, 10 mg/kg with dual administration protocol showed optimized effect of MCI-186 on the serum level of CPK and aldolase (Fig. 2B), similar to AST and LDH (data not shown); therefore, we chose this regimen in the following experiments.

With the use of serum samples obtained in animals at track 2, biomarkers indicating the muscular damage (CPK, AST, aldolase, and LDH) and renal function (BUN, K, and Cr) were determined (Fig. 2, C and D).

Serum concentrations of CPK, AST, aldolase (Fig. 2C), LDH (data not shown), BUN, and K (Fig. 2D) were significantly increased in the animals of the I/R and SeV-hFGF2 groups, and the increase of these markers was completely abolished in the MCI-186 group. There was no significant difference in the Cr concentration among the groups, but Cr concentration showed a similar tendency to that of the other parameters (data not shown). Additionally, we performed the same experiment with 6 h of ischemia before reperfusion as a preliminary study; however, >70% of animals died within the observation period (data not shown), suggesting that ischemia longer than 4 h might induce injury too severe for experimental evaluation.

These results thus indicate that the current animal model should be a relevant rat model of MNMS inducing extensive muscular and renal damage.

MCI-186, but not FGF-2, inhibits muscular edema. Next, we evaluated the edema of muscular tissue at track 2 because tissue edema has been reported as an important indicator of I/R injury (11).

As shown in Fig. 3A, histopathological sections showed apparent interstitial edema in the I/R and I/R + FGF-2 groups, and the effect was largely diminished by MCI-186 treatment. Furthermore, the weight-to-dry weight ratio was significantly increased in the animals of the I/R and SeV-hFGF2 groups, and this effect was almost completely inhibited by MCI-186 (Fig. 3B).

MCI-186, but not FGF-2, inhibits neutrophil infiltration to muscles and lungs. We next examined the neutrophilic infiltration into the local (right gastrocnemius muscle) and distant (right lung) organs by immunohistochemical labeling of MPO using samples obtained at track 2 because neutrophils have been suggested to play a significant role in the progression of I/R injury (1, 38).

As shown in Fig. 4, A and B, I/R caused marked infiltration of neutrophils in both muscles and lung, but neither was affected by FGF-2 gene transfer. In contrast, the number of MPO-positive neutrophils was dramatically diminished by MCI-186 treatment in both organs.

MCI-186, but not FGF-2, inhibits serum sICAM-1 level without a significant effect on a neutrophil chemoattractant. To seek the possible mechanism of the preventive effect of MCI-

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186 in the rat model of MNMS, we focused on the neutrophils and their infiltration pathway because local accumulation of neutrophils has been shown to play a significant role in the progression of I/R injury (3).

We first examined the number of neutrophils in the blood by using track 3 animals. As shown in Fig. 5A, a dramatic decrease in the whole WBC number was found in groups 2–4, suggesting the absorption due to the recruitment of WBCs into

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**Fig. 2.** Expression of intramuscular FGF-2 by ELISA (**A**), determination of the timing and dose of MCI-186 (**B**), and serum levels of biomarkers for muscular (**C**) and renal (**D**) damage. 

**A:** Intramuscular level of FGF-2. The right gastrocnemius muscular samples in track 1 were obtained 6 h after reperfusion and subjected to an ELISA that recognizes both murine and hFGF-2. *P < 0.01 vs. other groups. 

**B:** initial optimization study assessing the timing and dose of MCI-186. Dual administration of MCI-186 at 10 mg/kg showed optimized effect on the serum level of creatine phosphokinase (CPK) and aldolase.

**C:** serum levels of biomarkers for muscular damage in track 2 animals. AST, aspartate aminotransferase; GUT, glutamic-oxaloacetic transaminase. *P < 0.01 vs. groups 2 and 3; #P < 0.01 vs. group 1.

**D:** serum levels of biomarkers for renal damage in track 2 animals. BUN, blood urea nitrogen. *P < 0.01 vs. groups 2 and 3.
the reperfused and distant organs. However, the number of circulating neutrophils was not significantly different among groups tested (Fig. 5A, right).

Next, we measured the serum level of a neutrophil chemoattractant that is a rodent homolog of human IL-8, GRO/CINC-1, by using samples from track 2 animals. As shown in Fig. 5B, I/R strongly induced a rise in the serum level of GRO/CINC-1, which was not affected by any other treatment tested. Similar results were observed in the local protein content of GRO/CINC-1 in muscles from track 1 animals (Fig. 5B, right).

The above results taken together suggested that the preventive effect of MCI-186 did not include the proliferative and chemotactic processes of neutrophils. Therefore, we next assessed the expression of ICAM-1, which has been shown to be a major adhesion molecule for neutrophilic infiltration. We here examined the soluble form of ICAM-1 (sICAM-1) in the serum, because the serum level of sICAM-1 has been shown to reflect the expression of endothelial and membrane-bound ICAM-1 (16) and to be an important biomarker for endothelial function and inflammatory context (8, 9).

As shown in Fig. 5C, I/R significantly increased the serum level of sICAM-1, which was not affected by FGF-2 gene transfer. In contrast, treatment with MCI-186 significantly downregulated sICAM-1 at the control level, suggesting that the preventive effect of a free radical scavenger, MCI-186, to neutrophil infiltration might involve the suppression of the endothelial expression of ICAM-1.

**DISCUSSION**

Recent experimental studies have suggested the possible utility of angiogenic factors, including FGF-2, not only in the treatment of “tissue ischemia” (19, 21, 25, 32) but also “ischemia-reperfusion-mediated tissue injury” (12, 13, 24). We here demonstrated, however, the stronger protective effect of a free radical scavenger to the rat model of MNMS compared with that by FGF-2 gene transfer. These findings suggest the distinct molecular mechanism between tissue ischemia and I/R injury, implying that the scavenging of free radicals should be paid more attention than the restoration of blood perfusion when arterial reconstruction must be performed in the clinical setting.

An important advance obtained in this study is to demonstrate the information regarding the direct comparison of the effects of angiogenic therapy and the scavenging of radicals,
both of which were shown as effective, in a relevant rat model for MNMS. There is, however, still an unsolved issue to be clarified regarding the molecular and cellular mechanisms of free radicals in the progression of MNMS. We here attempted to address this issue in the current study.

One important question is what cell species are major targets of free radicals in MNMS. Because muscular edema has been shown as a result of microvascular permeability induced by vascular endothelial cell damage due to free radicals (14), vascular endothelial cells (ECs) would possibly be a critical determinant of free radical-induced tissue injury. This also suggests that MCI-186 may target ECs in the model used in the present study, and this is supported by the current results indicating the suppression of ICAM-1 expression that is specifically induced by ECs and, inversely, the lack of any effect on the expression of GRO/CINC-1 that is expressed by other cell types, including monocyte/macrophages (27). This is well supported by an in vitro experiment indicating the preventive effect of MCI-186 on vascular EC injury (35), so it is reasonable to conclude that vascular EC injury due to free radicals is the major cause of MNMS and that MCI-186 protects ECs from radical species.

The second question is whether the inhibition of neutrophil infiltration into the local and remote organs may be a cause or

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**Fig. 4.** Immunohistochemistry for myeloperoxidase (MPO) identifying neutrophilic infiltration into the local (muscle, A) and distant (lung, B) organs. Randomly selected 50 foci of high-powered view ($\times200$) per slide were counted under a light microscope, and the mean value was used as the MPO-positive cells/focus in each section. *$P < 0.01$ vs. groups 2 and 3; # $P < 0.05$ vs. group 1. **A:** MPO-positive neutrophil infiltration (arrows) into the local organ (muscle). A marked increase in the number of MPO-positive cells is evident in group 2 (I/R + PBS) and group 3 (I/R + FGF-2), a finding that is dramatically inhibited in group 4 (I/R + MCI-186). **B:** Similar results to A in the local organ (muscle) were obtained in the distant organ (lung).
a result of multiple organ failure in MNMS and of EC protection by MCI-186, as indicated by the reduced expression of sICAM-1. As shown in an important study using a liver injury model, a deficiency of ICAM-1 resulted in reduced liver injury associated with a decreased neutrophilic infiltration (6), suggesting that recruitment of neutrophils into the inflammatory foci via ICAM-1 is the major cause of tissue injury. From this point of view, anti-ICAM-1 therapy, including EC protection by MCI-186, might be an important strategy for the rescue of MNMS via reducing neutrophil-EC interactions.

The biological role of the increase of sICAM-1 in inflammatory diseases has not been well understood. As indicated in a number of studies, serum sICAM-1 level has been shown to correlate well with the EC expression of membrane-bound ICAM-1 (8, 9, 16), while sICAM-1 functions as a decoy for leukocyte adhesion inhibiting leukocyte-EC interaction (15, 28). Our preliminary study could not show the direct inhibitory effect of MCI-186 on ICAM-1 as well as sICAM-1 expression of human umbilical ECs stimulated by LPS and TNF-α in vitro, suggesting that the reduction of sICAM-1 in vivo has only an indirect effect on ECs. It has been demonstrated that membrane-bound ICAM-1 could be cleaved by metalloproteases, and, therefore, the reduction of the protease activity of such molecules should be involved in the effect of MCI-186. Further studies are called for to clarify this point further.

Considering a clinical setting, we have performed some additional experiments with later administration of MCI-186 after reperfusion in the present rat model of MNMS; however, very little effect was shown (data not shown), indicating that pretreatment of a free radical scavenger would be absolutely necessary to obtain a sufficient preventive effect on I/R injury in lower limb ischemia. Therefore, this drug should be administered, at least, just before reperfusion.
We here demonstrated that FGF-2 gene transfer was not effective and not toxic to the acute phase (6 h after reperfusion) after I/R injury of severe hindlimb ischemia; however, its benefit during the chronic phase has not yet been evaluated. The distinct mechanism between a free radical scavenger and FGF-2 gene transfer on limb ischemia may possibly show the additive effect on the chronic phase of the disease. Because such information is important in the clinical setting, we are now assessing this as a subsequent experiment.

In summary, we here demonstrated that free radicals played a critical role in the rat model of MNMS, a distinct mechanism from hindlimb ischemia. The beneficial effect of a free radical scavenger, but not FGF-2 therapy, was due to the inhibition of neutrophilic infiltration within local and remote organs, probably via inhibiting expression of sICAM-1. Therefore, the scavenging of free radicals generated after reperfusion should be paid more attention than angiogenesis when arterial circulation is reconstructed.

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