Transfection With a Dominant-Negative Inhibitor of Monocyte Chemoattractant Protein-1 Gene Improves Cardiac Function After 6 Hours of Cold Preservation

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Background—Monocyte chemoattractant protein-1 (MCP-1), a potent chemotactic factor for monocytes, is induced during ischemia-reperfusion. As monocytes might play an important causative role in reperfusion injury, we investigated if inhibition of monocyte activation could attenuate ischemia-reperfusion injury and thereby improve cardiac preservation. To inhibit monocyte activation, we transfected a dominant-negative inhibitor of MCP-1 (7ND) gene in an animal model.

Methods and Results—We used an isolated rabbit heart preparation perfused with support-rabbit blood and transfected 7ND genes to skeletal muscle of the support rabbits (n = 7) using electroporation technique; causing an elevation of serum 7ND level to 20 ± 7 pg/mL at 5 days after transfection. Animals receiving empty plasmid served as controls (n = 7). Five days after transfection, hearts from other rabbits were excised, stored in UW solution for 6 hours, and perfused with blood from transfected support rabbits. The 7ND group showed better cardiac output (128.7 ± 17.9 versus 81.6 ± 19.8 mL/min; P < 0.01), lower serum CK-MB levels (5.0 ± 1.8 versus 11.1 ± 2.9 ng/mL; P < 0.01), lower serum IL-1β levels (257.2 ± 23.2 versus 311.2 ± 37.4 pg/mL; P < 0.05), and lower serum TNF-α levels (19.0 ± 8.4 versus 35.1 ± 13.0 pg/mL; P < 0.05). The numbers of infiltrating cells in myocardium were significantly reduced in the 7ND group.

Conclusions—Inhibition of MCP-1 with 7ND gene transfection reduced cytokine activation, attenuated myocardial damage, and improved cardiac function after 6 hours of preservation. These results show that MCP-1 plays an important role in ischemia-reperfusion injury. (Circulation. 2003;108[suppl II]:II-213-II-218.)

Key Words: gene therapy • leukocytes • reperfusion injury • cytokines

Myocardial ischemia-reperfusion injury is believed to be associated with inflammatory reactions involving various types of cells and cytokines.1–3 Leukocytes are recognized as a major factor in ischemia-reperfusion injury. A number of studies, most focusing on neutrophils, have demonstrated harmful effects of leukocytes, such as the production of cytokines and oxygen radicals, adhesion to endothelium, and activation of complement systems.

Recently, in addition to neutrophils, several studies have demonstrated that in the early phase of reperfusion monocyte chemoattractant protein-1 (MCP-1) is induced and monocytes migrate into the reperfused myocardium.4–6 We thus hypothesized that inhibition of monocyte activation would attenuate ischemia-reperfusion injury.

To inhibit monocyte activation we used a 7ND skeletal muscle transfection model.7–11 7ND is an N-terminal deletion mutant of the MCP-1 gene and has been shown to be a dominant negative inhibitor of MCP-1.12,13 Studies show that 7ND protein is secreted from transfected skeletal muscle cells into circulating blood, and subsequently blocks MCP-1-induced chemotaxis in remote organs.7–11

Using a blood perfused isolated rabbit heart preparation,14,15 we investigated whether the inhibition of monocyte activation with 7ND gene transfer could attenuate ischemia-reperfusion injury.

Methods

Experimental Animals
Japanese white rabbits (KBT oriental, Tokyo, Japan) weighing from 2.9 to 3.3 kg were used (27 for preliminary experiments, and 14 each as heart donors, support rabbits, and blood donors). This experiment was reviewed by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Kyushu University, and was carried out under the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and the Law (No. 105) and Notification (No.6) of the Japanese Government.

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Figure 1. Line graph shows the levels of 7ND protein in serum after 7ND gene transfection. Levels significantly increase at 5 day. *P<0.01 versus before. All values are mean±SD (n=5).

Expression Vector Mutant

Human 7ND cDNA was constructed by recombinant polymerase chain reaction using a wild-type MCP-1 cDNA as template and cloned into the Bam HI (5') and Not I (3') site of the pcDNA3 expression vector plasmid.

Gene Transfer

Rabbits received intramuscular injections of empty plasmid or pcDNA3–7ND plasmid DNA (500 µg/kg) into the femoral muscle. Transgene expression was enhanced by intramuscular electroporation at the injection site immediately after injection. Six electronic pulses of 50V, 100 ms were applied to each injection site using an electric pulse generator (CUY201; BEX, Tokyo, Japan).

To determine the optimal timing for the experiment of ischemia-reperfusion, we performed preliminary experiments to examine at what day the 7ND protein level peaked after 7ND injection. We measured serum 7ND protein levels before, 3, 5, and 7 days after gene transfection by enzyme linked immunoassay using human MCP-1 ELISA kit (BioSource International, Inc., Camarillo, CA). Because this human MCP-1 ELISA kit does not react with the rabbit MCP-1, it was considered that serum 7ND protein levels could be measured by the use of this human ELISA kit. The serum 7ND protein levels peaked at 5 days after 7ND gene transfection (±7 pg/mL) (Figure 1). The rabbits injected with empty plasmid showed no detectable 7ND protein in the serum.

To confirm the effect of 7ND, recombinant human MCP-1 (2 µg/100 µL) or PBS (100 µL) was injected into the dermis 5 days after gene transfer to confirm the effect of 7ND. Twenty-four hours after the intradermal injection of MCP-1, a marked infiltration of mononuclear cells was detected in the MCP-1 injected site of rabbits that received injections of empty plasmid (Figure 2A). On the other hand, few infiltrated mononuclear cells were detected in the MCP-1 injected site of rabbits that received injections of 7ND genes (Figure 2B). At the PBS injected skin sites, no infiltration cells were detected (Figure 2C). Based on the data gained from these experiments, we decided to perform the main ischemia-reperfusion experiments 5 days after transfection of support rabbits with 7ND.

Donor Heart Management

The rabbits were anesthetized using sodium thiamylal (20 mg/kg) and intubated with a tracheal tube connected to a mechanical ventilator (model SN-480 to 5, Shimano, Tokyo, Japan) utilizing 100% oxygen. For further anesthesia, vecuronium bromide (1 mg/kg) and fentanyl citrate (700 µg/kg) were given. After performing a median sternotomy, the thymus and the pericardium were carefully removed and then the heart and aortic arch exposed. After heparinization (1500 U/kg), the common carotid artery and the external jugular vein were exposed and cannulated. Oxygenated blood from the common carotid artery of the support rabbit was introduced by a microtube pump (model MP-3, Tokyo Rikakikai Inc, Tokyo, Japan) to a cannula connected to the ascending aorta of the donor heart. The blood draining from the system was then returned to the jugular vein by another microtube pump (Figure 3A). During use of this system, hematocrit of perfusion blood was maintained at 23% by the using blood donor rabbits. Arterial blood gases analyses of the support rabbit were done with a pH/blood gas analyzer (model IL-1304, Instrumentation Laboratory, Barcelona, Spain). The femoral artery pressure of the support rabbit was also continuously monitored.

Measurement of Left Ventricular Function in Working Model

We measured LV function after 6-hours of preservation using a working preparation, as previously described.14 The donor heart was perfused through the aorta and then Langendorff preparation perfusion was initiated (Figure 3A). Perfusion pressure was maintained at 60mmHg and blood temperature maintained at 37°C. The superior and inferior vena cava and pulmonary veins were closed, and a double lumen cannula was inserted into the left atrium. One lumen of the double lumen cannula was connected to a pressure transducer to measure left atrial pressure (LAP), and the other to an atrial reservoir. Aortic flow rate was measured by an in-line electromagnetic flow probe (model 2N764, Transonic System Inc, NY) connected to a flowmeter (model T206, Transonic System Inc). Aortic pressure was measured from a sidearm in the aortic tract (Figure 3B). All signals (pressures and flows) were continuously monitored on an oscillograph (polygraph 360 system, NEC Sanei, Tokyo, Japan), digitized on-line at 200Hz with an analog-to-digital converter (MacLab System, AD Instruments, Ltd, Dunedin North, New Zea-
land) and recorded on a digital computer (PowerBook 550C, Apple Computer, Inc, Cupertino, CA).

In the working preparations, hearts were paced atrially at 250 beat/min and aortic afterload pressure fixed at 60 mm Hg. Aortic flow rates were measured at points of varied LAP. Systolic and diastolic pressures, the variables determined by the cardiac output and the stiffness or compliance of the afterload system, varied according to increases in LAP; whereas mean pressure was kept stable (60 mm Hg) throughout the measurements. Based on these data, we determined the Frank-Starling curve.

**Experimental Protocol**

The rabbits were divided into two groups according to the transfection of 7ND genes. The 7ND group (n=7) was transfected with pcDNA3–7ND plasmid DNA (500 µg/kg) and the control group (n=7) was transfected with the empty plasmid. Support rabbits and blood donors were transfected into their femoral muscle.

Five days after transfection, the heart from another rabbit was excised and stored in UW solution for 6 hours. After the preservation period, the heart was perfused with blood from a transfected support rabbit with the Langendorff preparation at 60 mm Hg of perfusion pressure. We measured the serum creatine kinase MB (CK-MB), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) levels in the coronary effluents at 10, 60, and 120 minutes after reperfusion. At 40 and 120 minutes after reperfusion, we measured left ventricle function in working preparation. The donor heart was paced atrially at 250 beat/min. After the measurement at 120 minutes after reperfusion, the donor heart was quickly removed from the cross-circulation system and the left ventricles were immersed in 10% paraformaldehyde histopathological examination. For a reference, we performed an experiment in which freshly harvested hearts without preservation were attached to the system using a nontransfected support rabbit (n=3). The support rabbit was killed with a lethal dose of anesthesia.

**Measurement of CK-MB, IL-1β, and TNF-α**

The serum CK-MB levels were measured by chemiluminescent immunoassay, and the IL-1β and TNF-α levels by enzyme linked immunoassay using a rat IL-1β and TNF-α ELISA kit (BioSource International, Inc).

**Histopathology and Number of Infiltrating Cells in Myocardium**

Cardiac tissue was fixed in 10% paraformaldehyde. Tissue was dehydrated, embedded in paraffin and cut into 5 µm thick slices, and then stained with Hematoxylin and Eosin. The number of infiltrating cells in myocardium was counted as the sum of the cell counts on 3 fields at ×400 magnification.6

**Statistical Analysis**

Results for each group are expressed as mean±SD. Values between the two groups were examined by two-way repeated measures

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**Figure 4.** Frank-Starling curve data obtained in this study. The red thick line represents the fitted Frank-Starling curve in the 7ND group (n=7). The red dashed lines show the individual curves in the 7ND group. The blue thick line represents the fitted Frank-Starling curve in the control group (n=7). The blue dashed lines show the individual curves in the control group. The black thick line represents the fitted Frank-Starling curve of freshly harvested hearts without preservation and gene transfection (n=3). (A) Frank-Starling curve data obtained at 40 minutes after reperfusion. The curve was significantly shifted to the left side in the 7ND group (y=−0.98x^2+28.82x−32.32) compared with that in the control group (y=−1.70x^2+35.11x−51.38). For reference, we also show fitted curves of freshly harvested hearts without preservation and gene transfection (y=−3.41x^2+55.41x−38.82). (B) Frank-Starling curve data obtained at 120 minutes after reperfusion. The curve was significantly shifted to the left side in 7ND group (y=−2.46x^2+37.08x−9.15) compared with that in control group (y=−2.30x^2+40.82x−67.88). We show fitted curves of freshly harvested hearts (y=−3.41x^2+55.41x−38.82). AoF=aortic flow, LAP=left atrial pressure.
analysis of variance (ANOVA). When ANOVA showed a significant intergroup difference, unpaired Student’s t test was used to examine the difference in each parameter. The relationship between LAP and AoF was analyzed by a multiple regression model using a dummy variable technique to investigate intergroup difference.17 Significance was designated as a probability value of less than 0.05.

Results
Between the two groups no significant differences were observed in body weights of donor rabbits. During the experiments, hemodynamics of the support rabbits were stable and systolic blood pressure was maintained at over 100 mm Hg. Minimum alterations in the arterial carbon dioxide tension, pH, and arterial bicarbonate levels were observed in the support rabbits.

Cardiac Function: Frank-Starling Curves
At both 40 and 120 minutes after reperfusion, the fitted Frank-Starling curve obtained by a multiple linear regression model was significantly shifted to the left ($P<0.01$) in the 7ND group compared with that of the control group (Figure 4). We also show fitted curves of freshly harvested hearts without preservation and gene transfection in Figure 4.

Serum Levels of CK-MB, IL-1β, and TNF-α
Serum CK-MB levels in the 7ND group were significantly lower than those in the control group at 120 minutes after reperfusion (5.0±1.8 versus 11.1±2.9 ng/mL, $P<0.01$) (Figure 5A).

Serum IL-1β levels in the 7ND group were significantly lower than those in the control group at 10 minutes (244.7±46.2 versus 300.6±37.8 pg/mL, $P<0.05$) and 60 minutes after reperfusion (257.2±32.3 versus 311.2±37.4 pg/mL, $P<0.05$) (Figure 5B).

Serum TNF-α levels in the 7ND group were significantly lower than those in the control group at 10 minutes after reperfusion (35.1±13.1 versus 19.0±8.4 pg/mL, $P<0.05$) (Figure 5C).

Histopathology and Number of Infiltrating Cells in Cardiac Tissue
Histological appearances of myocardium from the 2 groups are shown in Figure 6. The extravasation of polymorphonuclear and mononuclear leukocytes was recognized in the myocardium from the control group (Figure 6A). On the other hand, few leukocytes existed in myocardium from the 7ND group.
Discussion

In this study we demonstrated that inhibition of MCP-1 with 7ND gene transfection reduced cytokine activation, attenuated myocardial damage and improved cardiac function after 6 hours of cold preservation. Our results indicated that monocytes and their chemoattractant, MCP-1, played an important role in ischemia-reperfusion.

MCP-1 is a member of a family of proinflammatory cytokines called chemokines, which are structurally related. Chemokines affect the migration and activation of various types of leukocytes. MCP-1, a prototypic chemokine belonging to the C-C family, binds to MCP-1 receptor (CCR2) on monocytes as a dimer and exhibits chemoattractant potential for monocytes, but not for neutrophils. It has been demonstrated that MCP-1 expression is increased in chronic inflammatory diseases such as atherosclerosis and rheumatic arthritis. As has the migration of monocytes and inducement of MCP-1 at an early phase of reperfusion. Kumar et al have reported that MCP-1 mRNA was induced in endothelium of the small veins in ischemic and reperfused canine myocardium. Matsumori et al reported that plasma concentrations of MCP-1 are increased in patients after acute myocardial infarction. These studies indicate that increased MCP-1 expression is associated with monocyte accumulation. MCP-1 is a prototype of monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a member of a family of proinflammatory cytokines called chemokines, which are structurally related. Chemokines affect the migration and activation of various types of leukocytes. MCP-1, a prototypic chemokine belonging to the C-C family, binds to MCP-1 receptor (CCR2) on monocytes as a dimer and exhibits chemoattractant potential for monocytes, but not for neutrophils. It has been demonstrated that MCP-1 expression is increased in chronic inflammatory diseases such as atherosclerosis and rheumatic arthritis. As has the migration of monocytes and inducement of MCP-1 at an early phase of reperfusion. Kumar et al have reported that MCP-1 mRNA was induced in endothelium of the small veins in ischemic and reperfused canine myocardium. Matsumori et al reported that plasma concentrations of MCP-1 are increased in patients after acute myocardial infarction. These studies indicate that increased MCP-1 expression is associated with monocyte accumulation.

In this study, we directly inhibited MCP-1 and prevented activation of monocytes in a myocardial ischemia and reperfusion model, and showed that inhibition of MCP-1 attenuated reperfusion injury and preserved post-ischemia cardiac function. Our finding of improved cardiac function with MCP-1 inhibition was in accordance with the study of Ono et al that showed the favorable effects of administering anti-MCP-1 antibody to reduce the infarct size in a rat model of ischemia-reperfusion.

Although the exact mechanism by which cardiac function is improved with inhibition of MCP-1 is not clear, it would be highly probable that reduced production of inflammatory cytokines with MCP-1 inhibition was the main factor attenuating ischemia reperfusion injury. Herskowitz et al showed that monocytes are the main source of inflammatory cytokines in a myocardial ischemia and reperfusion model in rats and pigs, respectively. Inflammatory cytokines such as IL-1β and TNF-α were demonstrated to possess negative inotropic effects, and these were also shown to have chemoattractant actions for neutrophils, which play an important role in ischemia-reperfusion. In our study, the number of infiltrating cells in myocardium was significantly reduced in the 7ND group. Infiltrated cells found in the control group were polymorphonuclear and mononuclear leukocytes, which indicated that 7ND reduced the number of polymorphonuclear leukocytes as well. These findings suggested that inflammatory cytokines produced by monocytes activated neutrophils and further promoted inflammatory reactions in ischemia-reperfusion. Since 7ND and MCP-1 do not exhibit direct effects on neutrophils, it is highly probable that monocytes play a crucial role in the cascade of ischemia-reperfusion injury.

We used 7ND genes to perform anti-MCP-1 therapy in this study. 7ND lacks amino-terminal amino acids 2 to 8 in the amino sequence of MCP-1, and forms a heterodimer with wild type MCP-1. This heterodimer has been shown to bind to CCR2 and to work as a dominant negative inhibitor of MCP-1. 7ND protein is produced by 7ND gene transfection to skeletal muscle and intensively inhibits monocyte infiltration. Although a logical strategy to inhibit MCP-1 would be to administer 7ND protein at the time of reperfusion, we used 7ND gene transfection to the skeletal muscle because 7ND protein was not available.

In this study, we used a mutant gene of human MCP-1 in a rabbit model. The amino acid sequence similarity of rabbit MCP-1 to human MCP-1 is high and it has been demonstrated that human MCP-1 has a high affinity for rabbit monocytes. Indeed, we showed in the present study that 7ND gene transfer inhibited monocyte infiltration by intradermal injection of human MCP-1 in rabbits. Because recombinant rabbit MCP-1 was not available, we were not able to prove that 7ND gene transfer blocks activity of rabbit MCP-1. This is a limitation; however, we proved that inhibition of human MCP-1 induced monocyte infiltration into rabbit dermis by 7ND gene transfer. Since the sequence of human MCP-1 and rabbit MCP-1 show high similarity, it is highly probable that human 7ND may inhibit rabbit MCP-1. In fact, other studies show that 7ND is effective in experiments using mice, rats, and monkey. We recently showed suppression of macrophage recruitment into injured rabbit carotid artery by 7ND gene transfer. Considering those results, we believe it is safe to assume that 7ND blocked the action of rabbit MCP-1.

We used a blood-perfused isolated working rabbit heart model developed in our laboratory. Compared with isolated heart preparations perfused with crystalloid solutions, our blood-perfused model is well suited to investigate the process of ischemia-reperfusion, which involves numerous cellular components such as leukocytes and platelets. A potential limitation of our preparation is that because it is in extra-corporeal circulation, which allows blood to contact foreign surfaces; running an extra-corporeal circulation per se might have activated inflammatory process. To investigate the influence of our cross-circulation system on inflammatory reactions, we performed preliminary experiments running extra-corporeal circulation with support rabbits attached but without connecting the donor heart to the preparation. These showed that there were no increases in serum cytokine levels or blood leukocyte counts. Thus, we assumed that the increases in IL-1β and TNF-α observed in the controls were the result of ischemia and reperfusion.

The drug delivery system used in this study is a limitation; currently, 7ND protein has not been manufactured, delaying further study on the clinical application of 7ND. We modeled our experiments for hypothermic storage, but further refinement is needed in 7ND administration for clinical application. One possibility is to manufacture 7ND protein and deliver it...
intra venously; thus, 7ND may be administered to patients undergoing heart transplantation or open-heart surgery.

In conclusion, inhibition of MCP-1 with 7ND transfection reduced cytokine activation, attenuated myocardial damage and improved cardiac function after 6 hours of cold preservation. These results indicate that MCP-1 plays an important role in ischemia-reperfusion injury. 7ND gene transfection might become a useful strategy for attenuating ischemia-reperfusion injury.

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