Overexpression of Interleukin-1 Receptor Antagonist Provides Cardioprotection Against Ischemia-Reperfusion Injury Associated With Reduction in Apoptosis

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Background—Interleukin-1 (IL-1) plays a role in mediating acute inflammation during ischemia-reperfusion (I/R) injury in the heart, which leads to both necrosis and apoptosis of cardiomyocytes. IL-1 receptor antagonist (IL-1ra) is known to inhibit the effects of IL-1α and IL-1β, resulting in attenuated inflammatory injury, and to protect cells from IL-1β–induced apoptosis in vitro. We hypothesized that IL-1ra overexpression would provide cardioprotection by reducing inflammation-mediated myocardial damage including apoptosis after I/R injury in vivo.

Methods and Results—Rat hearts were transfected with human secreted–type IL-1ra gene by intracoronary infusion of Hemagglutinating Virus of Japan liposome and were heterotopically transplanted. IL-1ra overexpression in these hearts was confirmed by enzyme immunoassay and immunohistochemistry. Myocardial tolerance of the transplanted heart was evaluated with the use of a novel system in which the heart, existing within the recipient’s abdomen, was given 30 minutes of ischemia by left coronary artery occlusion and 24 hours of reperfusion. Consequently, infarct size was decreased in IL-1ra–transfected hearts compared with control-transfected ones (26.9±3.2% versus 46.2±3.0%, P=0.001), corresponding to lower myocardial myeloperoxidase activity (2.20±0.69 versus 6.82±1.19 U/g wet wt, P<0.001) and decreased neutrophil infiltration in histological study. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling and DNA-laddering studies demonstrated that cardiomyocyte apoptosis was attenuated in IL-1ra–transfected hearts (21.4±3.3 versus 41.4±3.4%, P=0.002), correlating with reduced post I/R upregulation of Bax, Bak, and caspase-3.

Conclusions—IL-1ra introduced by gene transfection protected myocardium from I/R injury by attenuating the inflammatory response, which was associated with decreased apoptosis. This suggests a potentially important role of IL-1/IL-1ra in myocardial I/R injury and the value of IL-1ra-gene therapy for myocardial preservation. (Circulation. 2001;104[suppl I]:I-308-I-313.)

Key Words: interleukins ■ inflammation ■ reperfusion ■ apoptosis ■ gene therapy

Clarifying the mechanism of myocardial ischemia-reperfusion (I/R) injury and developing its treatment is still of major interest in the field of cardiology and heart surgery.1,2 It is well known that a series of proinflammatory as well as anti-inflammatory cytokines play an important role in the myocardial damage caused by I/R injury.3,4 One of the most potent proinflammatory mediators is the early-acting cytokine interleukin-1 (IL-1), whose actions are regulated by the structurally related IL-1 receptor antagonist (IL-1ra). This naturally occurring protein is reported to inhibit the effects of IL-1α and IL-1β by competing for type I and type II IL-1 receptors, resulting in significantly attenuated inflammation.5,6 A beneficial anti-inflammatory effect of IL-1ra administration has been demonstrated in various experimental models such as renal ischemic injury,7 pulmonary hypertension,8 and sepsis.9 In addition, Yang and colleagues10 showed that adenoviral-mediated gene transfection of human IL-1ra gene attenuated the ischemia-induced inflammatory response in mouse brain. Bandara and coworkers11 reported that intra-articular transplantation of lapin synoviocytes overexpressing human IL-1ra by retroviral gene transfer attenuated IL-1β-induced inflammation. Qu and colleagues12 showed that skeletal myoblasts transfected with IL-1ra gene demonstrated improved survival after grafting to skeletal muscle by decreasing IL-1–mediated inflammation. However, the feasibility or effect of IL-1ra upregulation in the heart remains unclear.

In addition, although it is also reported that IL-1ra attenuates IL-1β–induced apoptosis in various cultured cells including cardiomyocytes,13–15 the role of IL-1ra in myocardial
apoptosis in vivo remains unknown. In the present study, therefore, we investigated the hypothesis that overexpression of IL-1ra by gene transfection would reduce myocardial inflammation and subsequent apoptosis caused by I/R injury in vivo, leading to enhanced myocardial tolerance.

**Methods**

**Gene Construction and Gene Transfection Protocol**

Using the DNA sequence within the GenBank Accession Number X52015, we cloned the 534 bp of cDNA for human secreted-type IL-1ra (hsIL-1ra) by PCR by using a high-fidelity DNA polymerase, KOD (Display System) from a human PBL cDNA library. This was cloned into pcDNA3.1+ vector (CMV promoter/enhancer; Invitrogen). Gene transfer was mediated by intracoronary infusion of Hemagglutinating Virus of Japan (HVJ) liposome. Briefly, 200 μg DNA mixed with 64 μg high-mobility group 1 nuclear protein was encased in liposome composed of phosphatidylserine, phosphatidylcholine, and cholesterol. The liposome was then incubated with 30,000 U of inactivated HVJ producing 4 mL of HVJ liposome.

Male Lewis rats weighing 200 grams were systemically heparinized (200 USP units IV); the hearts were arrested with cold cardioplegia (St Thomas’ Formula II) and removed under anesthesia with sodium pentobarbital (50 mg/kg IP). Thirty-three hearts were infused with 1 mL of ice-cold HVJ liposome containing pcDNA3.1+ with hsIL-1ra cDNA through the coronary arteries, with the vena cava and pulmonary arteries ligated. The control hearts were infused with 1 mL of HVJ liposome containing pcDNA3.1+ without hsIL-1ra cDNA. After 10 minutes of incubation under increased intracoronary pressure on ice, the hearts were transplanted into the abdomens of Lewis rats weighing 250 grams. The aorta and pulmonary artery of donor hearts were exposed, a 6–0 prolene suture was passed around the left ventricle (LV) to be frozen in embedding medium. Then the LV was cut into 4 segments and frozen in embedding medium.

**Immunohistochemical Analysis and Enzyme Immunoassay for IL-1ra**

At day 4 after gene transfection, 4 IL-1ra–transfected and 4 control-transfected hearts were collected under anesthesia as described. The left ventricle (LV) was cut into 4 segments to be frozen in embedding medium, cut into 6-μm sections, and fixed in 3.7% formaldehyde. The sections were incubated in 0.1% Triton X-100 and 10% FCS, then with a 1:100 dilution of anti-human IL-1α monoclonal antibody (R&D Systems) for 60 minutes at room temperature and finally with a 1:100 dilution of FITC-conjugated secondary antibody (Sigma). Another 5 IL-1ra–transfected and 5 control-transfected hearts were removed and the LV cut into small pieces, frozen in liquid nitrogen, homogenized, sonicated, and centrifuged at 35,000 g for 15 minutes. After measurement of protein concentration by the Bradford protein assay method (Bio-Rad), the supernatants were subjected to quantitative sandwich electroimmunoassay with a kit for rat IL-1ra (R&D Systems) and assayed spectrophotometrically according to the company’s instructions.

**Left Coronary Artery Occlusion and Reperfusion**

At day 4 after gene transfection, abdomens of recipient rats were opened again under the same anesthesia. After the transplanted heart was exposed, a 6–0 prolene suture was passed around the left coronary artery (LCA) ~3 mm distal to its origin. After 30 minutes of occlusion, the snare was released for reperfusion. The surgical wounds were repaired and the rats returned to the cages to recover.

**Systolic blood pressure of recipient rats was stable at between 105 to 120 mm Hg during the procedures.**

**Measurement of Infarct Size**

At 24 hours of reperfusion, the abdomen was reopened and the hearts again subjected to LCA ligation with the same sutures left in situ. Immediately after ligation, 1.5 mL of 2% Evans blue dye was injected through the recipient’s inferior vena cava to estimate the area perfused by the occluded artery. The dye infused through this route entered the systemic circulation of the recipient rat and stained every organ including the transplanted heart within a minute. For 6 hearts in each group, the LV was cut into 5 segments parallel to the apex-base axis, which were incubated with 1% triphenyltetrazolium chloride. The ischemic, infarcted, and nonischemic areas were separated and weighed. The infarct size was defined as the percentage of the wet weight of the infarct region to that of the ischemic region.

Another 12 hearts from each group were removed after Evans blue staining, and the ischemic regions were minced and frozen for analysis of myeloperoxidase (MPO) activity (n = 6) or DNA/protein assay (n = 6). For histological study, another 6 hearts were removed after Evans blue staining, and the ischemic regions were cut into 4 segments and frozen in embedding medium.

**Evaluation of Inflammatory Response**

MPO activity was measured as an index of neutrophil accumulation. The frozen samples of ischemic region were dissolved in 6 mL of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, homogenized, and centrifuged. MPO activity was measured by mixing 0.1 mL of the supernatant and 2.9 mL of 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL of dianisidine hydrochloride (Sigma) and 0.0005% H2O2. Absorption at 460 nm was read by a spectrophotometer (Beckman). Results are expressed in units of MPOμg of wet weight, each of which was defined as the enzymatic activity that degrades 1 μmol of H2O2/min at 25°C. For histological assessment of the inflammatory injury, the embedded samples were cut into 10-μm sections, fixed in 3.7% formaldehyde, and stained with hematoxylin and eosin.

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Staining**

Cryosections (6 μm) of ischemic regions were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)19,20 with a kit from R&D Systems. In brief, after the fixation described above, the samples were permeabilized and immersed in 5% H2O2. Residues of digoxigenin-nucleotide were then catalytically added to the 3′-OH ends of double- or single-stranded DNA by means of terminal deoxynucleotidyl transferase. After incubation with the anti–digoxigenin-peroxidase, color development was performed with a diaminobenzidine substrate/H2O2. Cell type was identified by counterstaining with 1% neutral red. The number of TUNEL-positive cardiomyocytes was divided by the total number of cardiomyocytes to determine the ratio of TUNEL-positive myocytes. Ten different fields for each section were analyzed.

**DNA Ladder and Western Blotting for Apoptosis-Related Proteins**

Frozen tissue samples were homogenized in an equal volume of homogenization buffer (10 mmol/L Tris-HCl, 25 mmol/L EDTA, 100 mmol/L NaCl, pH 8.0). For assessing DNA laddering,19,20 the homogenate (100 μL) was mixed with 1.25 mL of lysis buffer (10 mmol/L Tris-HCl, 25 mmol/L EDTA, 100 mmol/L NaCl, 1.0% SDS). The suspension was centrifuged at 13 000g for 15 minutes, and the supernatant was treated with 100 μg/mL proteinase K for 30 minutes at 50°C. The DNA was precipitated by adding ethanol and NaCl, dissolved in TE buffer, and extracted with phenol/chloroform. DNA extract was dissolved in 50 μL TE buffer and treated with RNase (100 μg/mL) for 30 minutes at 37°C. DNA aliquots (25 μg) were subjected to electrophoresis on 1.4% agarose gels.
To study apoptosis-related protein levels by Western blotting, the homogenate was sonicated and centrifuged at 35,000 g for 15 minutes. For the pre-I/R group, Western blotting was performed with the protein lysates that had been used for electroimmunoassay described above; 100 μg of protein was loaded onto a 10% SDS polyacrylamide gel for electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was blocked, incubated with a 1:1000 dilution of anti–Bcl-2, Bax, Bak, and caspase-3 polyclonal antibodies (Santa Cruz) and incubated with an HRP-conjugated secondary antibody (Sigma). Hyperfilm enhanced chemiluminescence was exposed to blots treated with enhanced chemiluminescence solution (Amersham), developed in a film processor, and scanned with a Molecular Dynamics 300A laser densitometer. To allow comparison between the groups, data are shown as percent density of bands versus corresponding pre-I/R samples of control-transfected hearts.

**Statistical Analysis**
All values are expressed as mean ± SEM. The differences in the data simply between two groups were determined by a Student’s t test. Comparison between groups on Western blotting data were assessed by 1-way ANOVA followed by Bonferroni’s post hoc test. A value of P < 0.05 was considered statistically significant.

**Results**

**Operative Mortality**
Rat hearts were transfected with hs-IL-1ra or control gene by intracoronary infusion of HVJ liposome, transplanted into the recipient’s abdomen, and subjected to I/R injury by LCA ligation. No animals were lost as a consequence of the operative procedures or complications.

**Expression of IL1-ra Introduced by Gene Transfection**
At day 4 after transfection, enzyme immunoassay demonstrated a significant (P < 0.0001) overexpression of IL-1ra in the LV myocardial tissue in IL-1ra–transfected hearts compared with control-transfected hearts (Figure 1A). Further, immunohistochemical examination with anti-human IL-1ra antibody demonstrated that the high-level IL-1ra was globally distributed throughout the LV myocardium of the IL-1ra–transfected hearts compared with those of control-transfected hearts (Figure 1, B and C).

**Infarct Size and Inflammatory Response After I/R Injury**
Infarct size caused by 30-minute LCA occlusion and 24-hour blood reperfusion was significantly (P = 0.001) decreased in IL-1ra–transfected hearts as compared with control-transfected hearts (Figure 2A). There was no significant difference in size of ischemic region, as a percentage to the total LV mass, between IL-1ra–transfected and control-transfected hearts (40.5 ± 3.4% versus 39.7 ± 2.9%).

MPO activity as an indicator of inflammatory response after I/R injury in the ischemic region of IL-1ra–transfected hearts was reduced to less than one third of that found in control-transfected hearts (Figure 2B). In addition, hematoxylin and eosin staining showed that numerous polymorphonuclear leukocytes (PMNL) had infiltrated the myocardium, occasionally forming a focus of infiltrating PMNL in the ischemic region of the control-transfected hearts (Figure 2C).
In contrast, these changes were markedly attenuated in the IL-1ra–transfected hearts (Figure 2D).

Myocardial Apoptosis Caused by I/R Injury
TUNEL staining demonstrated that the incidence of TUNEL-positive cardiomyocytes caused by 30 minutes of ischemia followed by 24-hour blood reperfusion was significantly reduced in IL-1ra–transfected hearts (B) compared with control-transfected hearts (A). Nuclei of TUNEL-negative cells are colored red. Bar, 50 μm. Incidence of apoptosis, defined as percentage of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes, is shown in graph (C). IL-1ra indicates IL-1ra-transfected hearts; control, control-transfected hearts (P<0.002 versus control; n=6 in each group; mean±SEM). DNA ladders were visible in agarose gels of DNA from the sample of control-transfected hearts, whereas ladders were not clear in those from IL-1ra–transfected hearts (D).

In contrast, these changes were markedly attenuated in the IL-1ra–transfected hearts (Figure 2D).

Figure 3. Myocardial apoptosis after I/R injury. After 30 minutes of ischemia followed by 24 hours of reperfusion, TUNEL staining demonstrated that incidence of TUNEL-positive cardiomyocytes (blue-stained nuclei) in ischemic area was reduced in IL-1ra–transfected hearts (B) compared with control-transfected hearts (A). Nuclei of TUNEL-negative cells are colored red. Bar, 50 μm. Incidence of apoptosis, defined as percentage of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes, is shown in graph (C). IL-1ra indicates IL-1ra-transfected hearts; control, control-transfected hearts (P<0.002 versus control; n=6 in each group; mean±SEM). DNA ladders were visible in agarose gels of DNA from the sample of control-transfected hearts, whereas ladders were not clear in those from IL-1ra–transfected hearts (D).

Discussion
We have, in this study, demonstrated that gene transfection mediated by intracoronary infusion of HVJ liposome can achieve high level IL-1ra expression in rat hearts and results in myocardial protection in terms of reduced infarct size after I/R injury induced by LCA occlusion and blood reperfusion. It has also been shown that this myocardial protective effect in IL-1ra–overexpressing hearts is associated with reduced inflammatory response evaluated by MPO activity and histological study as well as with attenuated cardiomyocyte apoptosis measured by TUNEL and DNA ladder studies. We consider that this protective effect of IL-1ra gene transfection shown in this study involves several interplaying mechanisms. Because it has been shown that IL-1 mediates the acute inflammatory response during I/R injury,3,4 one possible such mechanism is that myocardial overexpression of IL-1ra would directly inhibit the effects of raised IL-1 levels during I/R injury by competing for IL-1 receptors, resulting in attenuation of the inflammatory response and subsequent myocardial damage. This effect to attenuate inflammation would contribute to reduction in both myocardial necrosis and apoptosis caused by I/R injury. Second, it can be speculated that as IL-1–induced inflammation results in upregulation of IL-1, direct inhibition of the inflammatory response by IL-1ra might indirectly inhibit the rise in IL-1 level in turn, leading to reduction in IL-1–induced myocardial damage. In addition to the anti-inflammatory effect, we consider that the direct inhibitory effects of IL-1ra on IL-1β–induced apoptosis that may occur independent of I/R...
It has been shown that apoptosis of cardiomyocytes, which is involved in the process of myocardial damage after I/R injury, is associated with upregulation of Bax. Further, it has been demonstrated that IL-1β causes apoptosis of various cells including cardiomyocytes, associated with activation of Bak and Bcl-x; however, the mechanism by which I/R injury induces myocardial apoptosis in vivo is not fully understood. The present study has demonstrated that overexpression of IL-1ra, a potent inhibitor of IL-1β, attenuates myocardial apoptosis after I/R injury and is associated with reduced upregulation of Bax, Bak, and caspase-3 without affecting levels of anti-apoptotic Bcl-2. These data suggest that IL-1, presumably IL-1β, plays a significant role in myocardial apoptosis after I/R injury in vivo, which is related to activation of proapoptotic Bax and Bak, and that IL-1ra can in turn inhibit these adverse events mediated by IL-1β. Further investigation would be needed to clarify this mechanism in detail.

IL-1ra is a powerful anti-inflammatory cytokine, which could be expected to be useful as a clinical agent. We consider that strengthening the endogenous self-preservation system by genetic engineering is a promising advanced strategy to acquire a natural and higher level of myocardial protection. One of the advantages for gene transfer over recombinant protein administration is a persistent, long-term delivery of the protein. Raised myocardial levels of IL-1 are reported to occur for more than several weeks after I/R injury in rats. To address this, systemic delivery of recombinant protein has limitations that include the necessity of repeated injections of a large amount of peptide with possible side effects in other organs. Local drug delivery also has to be repeated with special equipment to ensure sustained high levels of IL-1ra in the myocardium. In contrast, gene transfection could effectively express the protein in the heart long enough to cover this period without repeated treatment. Thus, enhancing myocardial IL-1ra expression by gene transfection could be a promising, more advanced strategy for myocardial preservation.

It has also reported that increased levels of IL-1β are related to myocardial remodeling after infarction. IL-1β has been shown to inhibit phospholamban gene expression and induce hypertrophy in cultured cardiac myocytes. In addition, in vivo, expression of IL-1β correlates with the expression of extracellular matrix constituents including collagen types I and III in the postinfarction heart failure model, with a peak of IL-1β gene expression 1 week after infarction. Although, in the present study, we have investigated only the acute phase effect of IL-1ra overexpression after myocardial I/R injury, this overexpression, which is expected to last at least 2 to 3 weeks, might play a beneficial role in attenuating IL-1β-mediated adverse remodeling in the late phase of myocardial infarction. Further investigation is needed to evaluate this possible late phase effect.

It has been reported that intracoronary infusion of HVJ liposome can mediate gene transfection globally to the heart, with 50% to 80% of transfection efficiency in cardiomyocytes with little cellular damage. It has been demonstrated that hypothermia (4°C) is beneficial for attachment and adherence of HVJ liposome with cells and after normothermia (37°C) is advantageous in fusion of HVJ liposome into cells. After these processes, the introduced gene, enclosed within the HVJ liposome, is transferred to the cytosol and then immediately into nuclei with the help of high mobility group 1 nuclear protein. This translocation of the foreign DNA into the cell is mediated by the virus-cell fusion process, not through endocytosis. Therefore, the introduced gene is not degraded by the endosome or lysosome. In the present study, HVJ liposome retrogradely infused into the aorta of arrested, explanted hearts entered the coronary circulation globally, and these hearts were incubated on ice under increased intracoronary pressure. This pressure is supposed to be useful for HVJ liposome to permeate the endothelial barrier of small capillaries and achieve global distribution into the myocardial interstitium. Further, this process on ice would be useful in increasing the chance of adherence of HVJ liposome to the cells. After this step, the hearts were heterotopically transplanted to be reperfused with normothermic blood, which would be beneficial in cell–HVJ liposome fusion and transportation of the gene. Subsequent incubation for 4 to 5 days would allow the introduced gene to express proteins stably. This gene transfection system would be applicable to clinical settings of heart transplantation.

A common method for global gene transfection to the rat heart involves a series of steps to obtain high-transfection efficiency: infusion of a vector into the coronary arteries of excised hearts followed by heterotopic heart transplantation. Heart transplantation is required to allow the introduced gene to express proteins stably. For evaluating function or tolerance of these hearts, Langendorff crystalloid perfusion has been used to subject the hearts to I/R injury; however, it has several limitations such as the absence of neutrophils in the perfusate. Ex vivo blood perfusion is expensive and difficult to stabilize, requiring special equipment. We, in this study, have demonstrated the feasibility of an alternative, simple method to investigate myocardial tolerance against I/R injury in transplanted rat hearts existing within a recipient’s abdomen, whereby the hearts are subjected to ischemia by LCA occlusion and blood-reperfused by releasing the suture in situ in the abdomen. Mechanical ventilation is not necessary in this system. In addition, the results using this system, in terms of infarct size, MPO activity, and incidence of apoptosis, were consistent and comparable with those of the usual LCA occlusion model in rats, suggesting that this is a reliable and useful system for investigating myocardial tolerance of transplanted rat hearts by using blood perfusion.

In conclusion, we have shown that endogenous overexpression of IL-1ra mediated by gene transfection improves...
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myocardial tolerance to I/R injury and is associated with a reduction in apoptosis. This suggests a potentially important role of IL-1 and IL-1ra in myocardial I/R injury and highlights the value of IL-1ra gene therapy for myocardial preservation.

Acknowledgments

We would like to thank Dr Nobushige Yamashita (First Department of Internal Medicine and Department of Pathophysiology, Osaka University, Osaka, Japan) for his assistance in establishing the LCA occlusion technique.

References

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Circulation. 2001;104:I-308-I-313
doi: 10.1161/hc37t1.094871

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/104/suppl_1/I-308

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