Leukemia Inhibitory Factor Enhances Survival of Cardiomyocytes and Induces Regeneration of Myocardium After Myocardial Infarction

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Background—Myocardial infarction (MI) is a leading cause of cardiac morbidity and mortality in many countries; however, the treatment of MI is still limited.

Methods and Results—We demonstrate a novel gene therapy for MI using leukemia inhibitory factor (LIF) cDNA. We injected LIF plasmid DNA into the thigh muscle of mice immediately after inducing MI. Intramuscular injection of LIF cDNA resulted in a marked increase in circulating LIF protein concentrations. Two weeks later, left ventricular remodeling, such as infarct extent and myocardial fibrosis, was markedly attenuated in the LIF cDNA–injected mice compared with vehicle-injected mice. More myocardium was preserved and cardiac function was better in the LIF-treated mice than in the vehicle-injected mice. Injection of LIF cDNA not only prevented the death of cardiomyocytes in the ischemic area but also induced neovascularization in the myocardium. Furthermore, LIF cDNA injection increased the number of cardiomyocytes in cell cycle and enhanced mobilization of bone marrow cells to the heart and their differentiation into cardiomyocytes.

Conclusions—The intramuscular injection of LIF cDNA may induce regeneration of myocardium and provide a novel treatment for MI. (Circulation. 2003;108:748-753.)

Key Words: gene therapy ■ muscles ■ leukemia inhibitory factor ■ myocardial infarction ■ regeneration

The loss of cardiomyocytes in myocardial infarction (MI) is a major cause of heart failure. Many cardiomyocytes are dead by apoptosis and necrosis in MI, and the myocardium in the infarcted area is gradually replaced by collagen tissue. It has long been believed that adult cardiomyocytes do not proliferate; however, it was recently reported that adult cardiomyocytes could enter the cell cycle and increase the cell number. Moreover, it has been reported that cardiomyocytes can be generated from marrow stromal cells from mice in vitro and that undifferentiated stem cells in the bone marrow may be transported to the heart and differentiate into cardiomyocytes and vascular endothelial cells in vivo. Given these possibilities, MI could be treated by enhancing the ability of cardiomyocytes to divide or of bone marrow cells (BMCs) to differentiate into cardiomyocytes.

The interleukin 6 (IL-6) family of cytokines, including IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and cardiotoxin, have a variety of biological functions not only in the hematopoietic and immune systems but also in other organs, including the nervous and cardiovascular systems. The IL-6 family regulates growth and differentiation of many types of cells. Furthermore, these cytokines contribute to the regeneration of many tissues, such as nerves, skeletal muscle, liver, and bone. LIF has been reported to ameliorate denervation-induced muscle atrophy and improve regeneration of muscle and nerves. Locally administered LIF cDNA plasmid in a gelatin carrier can increase bone density and subsequent bone formation. In the heart, gp130, the common receptor of the IL-6 family, is expressed abundantly and has been reported to be critically involved in the growth and survival of cardiomyocytes.

Methods

Murine MI Model

MI was produced in 12-week-old male C57BL/6 mice by permanent ligation of the left coronary artery. A total of 40 mice were operated on to induce MI and randomly divided into 2 groups, LIF cDNA–injected and vehicle-injected groups (20 mice each). At 2...
days or 1 or 2 weeks after MI, 3 to 6 mice from each group were killed for analysis. The experiments were performed in accordance with the guidelines established by Chiba University for experiments in animals.

**Injection of Plasmids Carrying LIF cDNA**

The LIF expression vector was constructed by inserting a chimera of human and mouse LIF cDNA into the pCAGGS plasmid, which is driven by both a cytomegalovirus enhancer and a chicken β-actin promoter. The plasmid was prepared by use of the Concert High Purity Plasmid Purification Systems (Gibco BRL) and injected at a dose of 100 μg plasmids per 100 μL PBS per 20 g body wt into the thigh muscle of mice immediately after MI. The same volume of PBS alone was injected in littermate mice as controls.

**Assay of LIF Concentration**

Plasma LIF concentration was measured with a ELISA kit (Quantikine M) according to the manufacturer’s instructions. The LIF ELISA kit has high sensitivity by which the minimum 2 pg/mL of LIF can be detectable. The assay of this kit is specific for natural and recombinant mouse LIF.

**Echocardiography**

Transthoracic echocardiography was performed with an Agilent Sonos 4500 (Agilent Technologies Co) provided with an 11-MHz imaging transducer. When the mice had partially recovered from the light anesthesia, M-mode images of the left ventricle (LV) were recorded.

**Histology**

Hearts fixed in 10% formalin were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin-eosin and Azan. The extent of fibrosis was measured in 10 fields randomly selected from the LV free wall (LVFW) of each section. Five sections from each heart were measured, and the value was expressed as the ratio of Azan-stained fibrosis area to total myocardium area. The “remained myocardium” in the ischemic area was determined from 5 sections for each heart by calculating the ratio of (LVFW area – fibrotic area) divided by LVFW area.

**TUNEL Analysis**

Cardiomyocyte death was assessed in situ by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) of paraffin-embedded heart sections using Cardio TACS (Trevigen Inc).

**Isolation and Transplantation of BMCs**

BMCs were isolated from 8-week-old male transgenic mice (C57BL/6) systemically overexpressing green fluorescent protein (GFP), a kind gift from M. Okabe. BMCs (5 × 10⁷) suspended in 300 μL of RPMI medium were injected intravenously into each 8-week-old female C57BL/6 mouse whose total body had been irradiated (9 Gy) for 6 hours. Six weeks after BMC transplantation, the mice were operated on to induce MI. A flow cytometric analysis using an EPICS ALTRA (Beckman Coulter) equipped with a 525-nm filter at a bandwidth of ±15 nm revealed that >70% of BMCs were derived from donor cells.

**Immunohistochemistry and Western Blotting**

Paraffin-embedded cardiac sections were immunohistochemically stained by use of antibodies to Ki-67 (Dako), GFP (MBL, Japan), platelet and endothelial cell adhesion molecule (PECAM)-1, and cardiac troponin T (cTnT) (Santa Cruz) and MF20, and then with secondary antibodies conjugated with biotin, FITC, or rhodamine. Western blot analysis was performed with antibodies to vascular endothelial growth factor (VEGF) (Santa Cruz). Hybridizing bands were visualized by use of an enzyme-linked chemiluminescence detecting kit (Amersham Pharmacia Biotech).

**Fluorescence In Situ Hybridization for Y Chromosome**

Fluorescence in situ hybridization (FISH) for the Y chromosome was performed by use of the Y chromosome probe pUC18-Y351B, a kind gift from C.E. Bishop (Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Tex.). Engraftment of cardiomyocytes was assessed by FISH on paraffin-embedded cardiac sections after immunostaining for cTnT. Cardiac sections from normal male and female mice served as positive and negative controls, respectively.

**Statistical Analysis**

All values are expressed as mean±SD of 3 experiments in each instance. Comparisons were made by 1-way ANOVA followed by the Dunnett modified t test. Differences with a value of P<0.05 were considered statistically significant.

**Results**

**Increases in LIF Proteins After Injection of LIF cDNA**

Some cytokines, including the IL-6 family, have been reported to be increased in MI. We first examined LIF protein concentrations in blood of mice after inducing MI. Endogenous plasma LIF concentrations were increased transiently after MI (before, 0.06 ng/mL; 2 days, 2.1 ng/mL; 1 week, 0.8 ng/mL; 2 weeks, 0.4 ng/mL; 4 weeks, 0.13 ng/mL). In mice that were injected with LIF plasmid DNA into the thigh muscle immediately after MI had been induced, LIF protein was markedly increased. The concentrations of LIF peaked at 1 week (5.3 ng/mL) after the injection of LIF cDNA and remained persistently high for >4 weeks (1.4 ng/mL).

**Attenuation of Infarct Extent and LV Remodeling and Improvement of Cardiac Function by LIF**

To elucidate whether an increase in LIF protein has beneficial effects on MI, we assessed cardiac function and morphology by echocardiography at 2 weeks after MI. In vehicle-injected MI mice, the posterior wall thickness of the LV was decreased significantly compared with sham-operated mice, and the thickness of the interventricular septum was conversely increased (Table). Moreover, LV internal dimensions at both end-diastole and end-systole were increased, whereas the fractional shortening and ejection fraction were decreased (Table), suggesting that LV remodeling and cardiac dysfunction occurred after MI. In LIF cDNA–injected MI mice, all these changes were less prominent and cardiac functions were much better than in vehicle-injected mice (Table). In vehicle-injected MI mice, the infarct area was quite large and the LVFW was very thin (Figure 1A). Fibrosis was so prominent that there were only a few cardiomyocytes in the infarct zone (Figure 1, A and B). In contrast, in LIF cDNA–injected mice, the cardiac muscle was preserved and the LVFW was much thicker (Figure 1A). Fibrosis was less remarkable in the ischemic zone (Figure 1, A and B). All these findings suggest that LIF does exert beneficial effects on MI from the viewpoints of cardiac morphology and function.

**Protection Against Cardiomyocyte Death and Induction of Neovascularization by LIF**

Because LIF has been reported to protect cardiomyocytes from injury, we examined, by use of the TUNEL method,
whether LIF prevents cardiomyocytes from death at MI. Many TUNEL-positive cardiomyocytes were observed in the border zone of the infarcted heart (Figure 2A). The number of TUNEL-labeled cardiomyocytes was significantly less in LIF-treated mice than saline-injected mice (Figure 2, A and B), indicating that LIF protects cardiomyocytes from death after MI.

Myocardial ischemia is a major cause of cardiomyocyte death. Activation of gp130 signaling has been reported to promote neovascularization in the myocardium.28 We therefore examined whether LIF induced new vessel formation in the heart after MI. We used PECAM-1 as a marker for neovascularization.29,30 Immunohistochemical analysis using anti-PECAM antibody revealed that there was more newly formed vasculature in the border zone of LIF-treated mice than in vehicle-treated mice (Figure 3, A and B). Because VEGF is the most potent angiogenic factor,31 we examined the expression of VEGF protein in the myocardium after MI. At 1 week after MI, VEGF was slightly upregulated in the hearts of vehicle-treated mice, whereas a marked increase in VEGF was observed in the heart of LIF-injected mice (Figure 3C). These findings suggest that LIF enhanced neovascularization.

**Figure 1.** Left ventricular remodeling after 2 weeks of MI. A, Cardiac morphology. Fixed hearts were stained with hematoxylin-eosin (H-E) and Azan. Representative photographs are shown. B, Size of remained myocardium and extent of fibrosis were measured as described in Methods. Data are shown as mean ± SD of 3 hearts. *P<0.05.

**Figure 2.** TUNEL analysis after 2 weeks of MI. A, Representative TUNEL staining of heart. Brown staining indicates TUNEL-positive nuclei. B, Number of TUNEL-positive cardiomyocytes counted in 20 fields randomly selected from LVFW of each section. Ten sections from each heart were measured; values are expressed as number of dead cells per mm² of LVFW area. Data are shown as mean ± SD of 3 hearts. *P<0.05.

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BW indicates body weight; HW, heart weight; IVSTD, interventricular septum thickness; LVFWT, LV posterior wall thickness; LVIDd and LVIDs, LV internal dimensions at end diastole and end systole, respectively; EF, ejection fraction; and FS, fractional shortening. Data are expressed as mean±SD.

*P<0.05 vs sham-operated mice.
†P<0.05 vs MI with PBS injection.
Differentiation of BMCs into cardiomyocytes was confirmed by use of immunohistochemical (IHC) staining with anti-PECAM antibody in hearts. Arrows indicate PECAM-positive vessels. B, Number of PECAM-positive vessels counted in 20 randomly selected fields from LVFW for each section. Ten sections per heart were counted; values are expressed as number of PECAM-positive vessels per mm² of LVFW. Data are shown as mean ± SD of 3 mice. *P<0.05. C, Expression of VEGF. Total proteins extracted from LV tissue excised at indicated time points after MI were separated in 12% SDS gels, and blotted membranes were incubated with anti-VEGF antibody or anti-α-actin antibody as an internal control. Representative autoradiograms from 3 independent experiments are shown.

Figure 3. Neovascularization after 2 weeks of MI. A, Representative photographs showing immunohistochemical (IHC) staining with anti-PECAM-positive vessels. B, Number of PECAM-positive vessels counted in 20 randomly selected fields from LVFW for each section. Ten sections per heart were counted; values are expressed as number of PECAM-positive vessels per mm² of LVFW. Data are shown as mean ± SD of 3 mice. *P<0.05. C, Expression of VEGF. Total proteins extracted from LV tissue excised at indicated time points after MI were separated in 12% SDS gels, and blotted membranes were incubated with anti-VEGF antibody or anti-α-actin antibody as an internal control. Representative autoradiograms from 3 independent experiments are shown.

Figure 4. Number of Ki-67-positive cardiomyocytes in cell cycle after 1 week of MI. Ki-67-positive cardiac nuclei were counted in 20 fields randomly selected from LVFW for each section. Twenty sections from each heart were measured. Values are expressed as number per mm² of LVFW area. Data are shown as mean ± SD of 3 mice. *P<0.05.

Proliferation of Cardiomyocytes and Differentiation of BMCs Into Cardiomyocytes

The IL-6 family of cytokines has been reported to promote growth and proliferation of many types of cells, including cardiomyocytes.9,15,17,19,20 We next examined whether LIF stimulated the proliferation of cardiomyocytes after MI. To identify the cardiomyocytes that had entered the cell cycle, we examined the expression of Ki-67 protein, which has been reported to be expressed only in the nucleus of proliferating cells.32,33 Only a small number of Ki-67-positive cardiomyocytes were recognized in the border zone of vehicle-treated mice (Figure 4), whereas in the LIF-injected mice, there were significantly more Ki-67-positive cardiomyocytes (Figure 4).

It was recently reported that undifferentiated stem cells in the bone marrow may be transported to the heart by cytokines and differentiate into endothelial cells and cardiomyocytes.7 We examined whether LIF stimulated the mobilization and differentiation of BMCs after MI. We transplanted BMCs isolated from GFP-transgenic mice into irradiated wild-type mice and induced MI in them. Two weeks after MI, there were a few GFP-positive cells in the border zone of the infarction in the vehicle-injected mice (data not shown). In the heart of LIF-injected mice, however, there were clusters of GFP-positive cells in certain areas (Figure 5B). Furthermore, there were more GFP-positive cardiomyocytes in the myocardium of LIF-injected mice than in that of vehicle-treated mice (Figure 5, C and D). Also, differentiation of BMCs into cardiomyocytes was confirmed by use of immunostaining for cTnT followed by Y chromosome FISH. After 2 weeks of MI, positive signals for Y chromosome were observed in the myocardium of LIF-injected female recipients that had received transplants of male BMCs (Figure 5E). More GFP-positive capillaries were also observed in the border zone of the infarcted heart of LIF cdNA–treated mice (PBS-treated, 11±4/mm²; LIF-treated, 23±3/mm²; P<0.05) (Figure 5F). These results collectively suggest that LIF induced mobilization and differentiation of BMCs into cardiomyocytes and vascular endothelial cells.

Discussion

LIF has potent effects on various cellular events such as proliferation, differentiation, and survival of many cells.10,16,18,34–36 Activation of gp130 by LIF activates several pathways, including the Jak/STAT-3, phosphoinositide-3'-kinase (PI3K)/Akt, and Ras/ERK pathways.18,35,37 Activation of the Jak/STAT-3 pathway has been reported to induce upregulation of antiapoptosis proteins such as Bcl-2, Bcl-xl, nuclear factor-κB, and SOCS.35 Activation of the PI3K/Akt and Ras/ERK pathways has been reported to enhance survival of many types of cells.18,37 The activation of these pathways in the heart may prevent cardiomyocytes from death in MI. Neovascularization is important to prevent further cell death and improve cardiac function during LV remodeling after MI.33,38 Activation of STAT-3 has been reported to enhance neovascularization in the myocardium through enhanced expression of VEGF.28 Our results also showed that LIF suppresses cardiomyocyte death and increases vasculature in the border zone of infarcted hearts through enhanced expression of VEGF.

Although cardiomyocytes have long been thought to be terminally differentiated and do not undergo mitosis after birth, it was recently reported that even adult mammalian cardiomyocytes can divide.3,33,39 However, their proliferative ability is so limited that the large infarcted area with fibrotic tissue results in cardiac dysfunction. The IL-6 family of cytokines has been reported to induce proliferation of many types of cells,9 and the results of knockout and dominant negative experiments suggest that gp130 is involved in the growth and proliferation of cardiomyocytes.15,17 It is interest-
ing to note that there were many cardiomyocytes that expressed Ki-67 in the ischemic zone as well as in the border zone in LIF-injected mice. Although it remains to be determined how many cardiac myocytes are increased, LIF-induced proliferation of cardiomyocytes may contribute, at least in part, to the increase in cardiomyocytes in the ischemic area of MI hearts.

Recently, BMCs have been reported to differentiate into cardiomyocytes after MI.\textsuperscript{5,6,8} It has also been suggested that pretreatment with stem-cell factor and granulocyte-colony-stimulating factor promoted the mobilization of BMCs into the myocardium and their differentiation into cardiomyocytes after induction of MI.\textsuperscript{7} Here, we also provide evidence that LIF induces not only mobilization of BMCs to myocardium but also differentiation of BMCs into cardiomyocytes after MI. Although LIF inhibits differentiation and maintains stem-cell renewal of embryonic stem cells in vitro,\textsuperscript{22} LIF induces differentiation of many cell types.\textsuperscript{11-14,40} The bidirec-
tional effects of LIF on differentiation are dependent on the cell lineages and stages of differentiation.\textsuperscript{40}

LIF protected cardiac myocytes from death and enhanced neovascularization after MI. LIF also induced an increase in cardiomyocytes that are in the cell cycle. Moreover, LIF promoted mobilization of BMCs into the myocardium and their differentiation into cardiomyocytes. These pleiotropic effects of LIF may contribute to attenuating the extension of infarction and improve cardiac function. It was recently reported that adult humans have extracardiac progenitor cells that mobilize into and regenerate damaged myocardium at very low levels.\textsuperscript{41} It remains to be determined whether LIF gene therapy promotes this process after MI in humans.

**Acknowledgments**

This study was supported by a Grant-in-Aid for Scientific Research, Developmental Scientific Research, and Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and

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**Figure 5.** Mobilization and differentiation of BMCs 2 weeks after MI. A, Representative staining of hearts from wild-type (top) and GFP-transgenic mice (bottom). B, GFP-positive cells in MI heart of LIF cDNA-injected mice. BMCs formed clusters in some areas of border zone. C, MI heart of LIF cDNA-injected mice. A GFP-positive cardiomyocyte was observed in border zone. Red fluorescence indicates cardiac myosin; green fluorescence indicates GFP-positive cells; yellow labeling indicates cardiomyocyte derived from GFP-positive cells. A–C, Left, stained with MF20 (anti-myosin antibody); middle, GFP; right, merge. D, Number of GFP-positive cardiomyocytes. Cardiomyocytes were counted in whole LVFW area for each section. Twenty sections from each heart were measured. Values are expressed as number of GFP-positive cardiomyocytes per 10\(^6\) cardiomyocytes. Data are shown as mean ± SD of 3 mice, \(P<0.05\). E, Representative FISH for murine Y chromosome and immunostaining for cTnT in border zone. Staining: green, cTnT; blue, DAPI for nuclear chromatin; red, rhodamine for Y chromosome. Cardiomyocyte nuclei contain Y chromosomes (arrows), indicative of BMC origin. F, Representative staining of GFP-positive microvessels in border zone. Red fluorescence indicates staining of PECAM; green fluorescence indicates GFP-positive cells; yellow labeling indicates newly formed vessels derived from GFP-positive cells (arrows). Arrowheads show a GFP-positive cell unlabeled by PECAM.
References
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Circulation. 2003;108:748-753; originally published online July 14, 2003;
doi: 10.1161/01.CIR.0000081773.76337.44

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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