Therapeutic Activation of Signal Transducer and Activator of Transcription 3 by Interleukin-11 Ameliorates Cardiac Fibrosis After Myocardial Infarction

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Background—Glycoprotein 130 is the common receptor subunit for the interleukin (IL)-6 cytokine family. Previously, we reported that pretreatment of IL-11, an IL-6 family cytokine, activates the glycoprotein 130 signaling pathway in cardiomyocytes and prevents ischemia/reperfusion injury in vivo; however, its long-term effects on cardiac remodeling after myocardial infarction (MI) remain to be elucidated.

Methods and Results—MI was generated by ligating the left coronary artery in C57BL/6 mice. Real-time reverse transcription polymerase chain reaction analyses showed that IL-11 mRNA was remarkably upregulated in the hearts exposed to MI. Intravenous injection of IL-11 activated signal transducer and activator of transcription 3 (STAT3), a downstream signaling molecule of glycoprotein 130, in cardiomyocytes in vivo, suggesting that cardiac myocytes are target cells of IL-11 in the hearts. Twenty-four hours after coronary ligation, IL-11 was administered intravenously, followed by consecutive administration every 24 hours for 4 days. IL-11 treatment reduced fibrosis area 14 days after MI, attenuating cardiac dysfunction. Consistent with a previous report that STAT3 exhibits antiapoptotic and angiogenic activity in the heart, IL-11 treatment prevented apoptotic cell death of the bordering myocardium adjacent to the infarct zone and increased capillary density at the border zone. Importantly, cardiac-specific ablation of STAT3 abrogated IL-11–mediated attenuation of fibrosis and was associated with left ventricular enlargement. Moreover, with the use of cardiac-specific transgenic mice expressing constitutively active STAT3, cardiac STAT3 activation was shown to be sufficient to prevent adverse cardiac remodeling.

Conclusions—IL-11 attenuated cardiac fibrosis after MI through STAT3. Activation of the IL-11/glycoprotein 130/STAT3 axis may be a novel therapeutic strategy against cardiovascular diseases. **(*Circulation*. 2010;121:684-691.)**

Key Words: interleukins ■ myocardial infarction ■ remodeling ■ signal transduction

After myocardial injury, various kinds of neurohumoral factors and cytokines modulate cardiac remodeling. Among them, leukemia inhibitory factor (LIF) and cardiotrophin-1, which belong to the interleukin (IL)-6 family, play important roles in cardioprotection.[1,2] LIF and cardiotrophin-1 are secreted from cardiomyocytes in response to pathological stress.[3–5] These cytokines bind and activate LIF receptor in cardiomyocytes.[6] Activated LIF receptor makes a dimer with glycoprotein 130 (gp130), followed by activation of signal transducer and activator of transcription 3 (STAT3).[7] STAT3 activation promotes cardiomyocyte survival and vascular formation in the heart.[8–10] Thus, cardiac activation of the gp130/STAT3 system may be a potential therapeutic strategy against cardiovascular diseases; however, therapies targeting gp130 have not been proposed.

Clinical Perspective on p 691

The difficulty in therapeutic activation of gp130 is derived from its receptor system. Gp130 is expressed ubiquitously as the common receptor subunit of IL-6 family cytokines.[11] IL-6 family cytokines bind their specific receptor α subunits, followed by activation of a common gp130 receptor. Pleiotropic effects of IL-6 family cytokines are explained by the differential expression of receptor α subunits. Most members of the IL-6 family, whose receptor α subunits are expressed abundantly in inflammatory cells, would evoke severe in-

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flammation[12] as a serious adverse event if administered systemically. Therefore, to achieve clinical use of IL-6 family cytokines, the cytokine that induces only a tolerable level of inflammation should be selected.

IL-11 is a hematopoietic IL-6 family cytokine with pleiotropic effects. IL-11 exhibits thrombopoietic activity, and recombinant human IL-11 is used clinically for thrombocytopenia.[13] In contrast to other IL-6 family members, IL-11 exhibits anti-inflammatory activity against chronic inflammatory diseases, such as Crohn disease.[14] Moreover, recombinant human IL-11 protects epithelial cells of the intestine from tissue damage, suggesting its cytoprotective property.[15] Recently, we demonstrated that the IL-11 receptor is expressed in cardiomyocytes and that pretreatment of IL-11 confers resistance to ischemia/reperfusion injury in a murine model as a preconditioning effect.[16] When the limited level of clinical adverse effects of recombinant human IL-11 is considered,[13] IL-11 may be a candidate to be available clinically as cardiac gp130-targeting therapy against heart diseases.

In this study, we investigated the long-term effects of IL-11 treatment after myocardial infarction (MI). In addition, we report that IL-11 treatment prevents adverse cardiac remodeling through the STAT3 pathway.

Methods

Animal Care

The care of all animals was in compliance with the Osaka University animal care guidelines. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1996).

Coronary Artery Ligation and IL-11 Treatment

MI was generated by coronary artery ligation according to the previous report[1] with minor modifications. Briefly, C57BL/6 mice (8 to 10 weeks old; Japan SLC) were anesthetized and ventilated with 80% oxygen containing 1.5% isoflurane (Merck). After left-side thoracotomy, the left coronary artery was ligated with 7-0 silk sutures. Infarction was confirmed by discoloration of the ventricle and ST-T changes on ECG. The chest and skin were closed with 5-0 silk sutures. In preliminary experiments, we confirmed that the operation reproducibly generates infarction with the initial area at risk 20% to 25% per left ventricular (LV) area, as analyzed by Evans blue exclusion assays.[16] Sham-operated mice were subjected to similar surgery, except that no ligature was placed. Twenty-four hours after MI operation, mice were randomly assigned to 2 groups: the IL-11 group and control group. In the IL-11 group, recombinant human IL-11 (Peprotech) was administered intravenously for 5 days consecutively; the control group received the same volume of phosphate-buffered saline (PBS) during the same period.

Real-Time Reverse Transcription Polymerase Chain Reaction

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed according to the manufacturer's protocol. Total RNA was prepared from hearts at various time points after operation. In some experiments, the hearts were cut into 2 pieces: infarct area and remote area. The infarct area is the damaged or fibrotic region with its surrounding border zone, and the remote area is the portion separated from the infarct area by >1 mm.

Total RNA (1 μg) was subjected to first-strand cDNA synthesis with oligo(dT) primer. IL-11 was quantified by real-time RT-PCR with the use of the ABI PRISM 7700 sequence detection system (Applied Biosystems). As an internal control, the expression of GAPDH mRNA was estimated with the SYBR green system. The primers for IL-11 or GAPDH are as follows: IL-11 forward: 5′-CTGCCCACCTTGGCCATGAG-3′; IL-11 reverse: 5′-CCAGGCGAGACATCAAGAAAGA-3′; GAPDH forward: 5′-GCCGGTGCTGAGTATGTCGT-3′; GAPDH reverse: 5′-CCCTTTTGGCTCCACCCTT-3′.

Immunoblot Analyses

Immunoblot analyses were performed as described previously.[17] Heart homogenates were prepared in buffer containing 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1% Triton X-100, 1% deoxycholic acid, and 1 mmol/L dithiothreitol. Proteins were separated by SDS-PAGE and transferred onto the polyvinylidene difluoride membrane (Millipore). The membrane was immunoblotted with anti-phospho-STAT3 (p-STAT3) (Cell Signaling Technology, Danvers, Mass), anti-Bcl-2 (BD Transduction Laboratories), anti-survivin (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), or anti-cleaved caspase 3 (Cell Signaling Technology) antibody. The membrane was reprobed with anti-STAT3 (Santa Cruz Biotechnology) or anti-GAPDH (Chemicon, Temecula, Calif) antibody to show equal amount loading.

Histological Estimation of Cardiac Fibrosis

The frozen sections (5-μm thick) were prepared from the portion $\approx$300 μm distal to the ligation point and stained with Masson's trichrome. Photomicrographs were taken, and fibrotic circumference and area were measured with the use of Scion Image (Scion Corporation) by a researcher who was blinded to the treatment. Fibrotic circumference and area were calculated as a percentage of LV circumference and area, respectively. Infarct wall thickness was measured perpendicular to the infarcted wall at 3 separate regions and averaged.

Analysis of Cardiac Function

Fourteen days after MI, mice were anesthetized (50 mg/kg pentobarbital) and heparinized (50 U) via intraperitoneal injection. The hearts were excised rapidly and placed in ice-cold modified Tyrode's solution (140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.45 mmol/L $MgCl_2$, 0.33 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5 mmol/L HEPES [pH 7.4]). The aorta was cannulated and retrogradely perfused at a constant pressure of 100 mm Hg with Tyrode's solution bubbled with 80% oxygen at 37°C. The experiments were performed at 37°C by immersing the heart in Tyrode's solution in a water-jacketed chamber. The hearts were paced at 420 bpm. The fluid-filled balloon was inserted into the LV to monitor cardiac function. The balloon was attached to a pressure transducer, which was coupled to a 4S PowerLab (AD Instruments). LV developed pressure and $\pm$dP/dt were measured.

Immunofluorescent Microscopic Analyses

The hearts were harvested 15 minutes after intravenous injection of IL-11, and the frozen sections were prepared. The sections were stained with anti-p-STAT3 and anti-sarcomeric α-actinin (Sigma) antibodies. Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa Fluor 546–conjugated goat anti-mouse IgG (Molecular Probes) were used as secondary antibodies. Nuclei were also stained with Hoechst 33258.

Apoptotic cell death was detected by a terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL) staining with an in situ Apoptosis Detection Kit (TaKaRa). The section was costained with anti-sarcomeric α-actinin antibody to identify the cardiac myocytes. For quantitative analyses, apoptotic myocytes were counted in number by a researcher who was blinded to the assay conditions.

Immunohistochemical Analyses

The frozen sections were prepared as described above. Capillary density was examined by immunohistochemical staining with the use of the Vectastain ABC kit (Vector Laboratories) with anti-CD31

density quantitatively, the number of capillaries was counted by a researcher who was blinded to the assay conditions.

Conditional Ablation of the *STAT3* Gene in Cardiomyocytes of Adult Murine Heart

The transgenic mice in which Cre recombinase fused to the mutated estrogen receptor domains (MerCreMer) were driven by the cardiomyocyte-specific α-myosin heavy chain (α-MHC) promoter, designated as α-MHC-MerCreMer mice, were a gift from Dr Molkentin.[18] We crossed the α-MHC-MerCreMer mice with mice that carried floxed STAT3 alleles ($STAT3^{flox/flox}$)[19] and produced α-MHC-MerCreMer/$STAT3^{flox/flox}$ mutant mice. To induce Cre-mediated recombination, mice were treated with 20 mg/kg tamoxifen (Sigma, St Louis, Mo) by intraperitoneal injection once per day for 5 consecutive days. Five days after the last treatment, the level of STAT3 expression decreased dramatically, and the mutant mice underwent MI as described above.

Cardiac-Specific Transgenic Mice Expressing Constitutively Active STAT3

Generation of cardiac-specific transgenic mice expressing constitutively active STAT3 was described previously.[9]

Statistical Analysis

Data are presented as mean±SD. Comparisons between 2 groups were performed with the use of the unpaired *t* test. One-way ANOVA with the Bonferroni test was used for multiple comparisons. Differences were considered statistically significant when the calculated (2-tailed) *P* value was <0.05.

Results

IL-11 Is a Cardiac Cytokine That Activates STAT3 in Cardiomyocytes In Vivo

We analyzed the expression of IL-11 mRNA in hearts at various time points after MI. Hearts were separated into infarct area and remote area, and the expression of IL-11 mRNA was measured by real-time RT-PCR (Figure 1A). The expression of IL-11 transcript was elevated, with its peak at 1 day after MI, and was gradually reduced at both infarct and remote areas. In the infarct area, the enhanced expression of IL-11 was sustained for 14 days. These data indicate that IL-11 is produced in the heart during cardiac remodeling after MI.

Next, we examined whether intravenous administration of IL-11 stimulates STAT3 in hearts by immunoblot analysis with anti-p-STAT3 antibody (Figure 1B). STAT3 phosphorylation was induced rapidly and reduced to the basal level within 180 minutes after IL-11 injection. IL-11 activated STAT3 in the heart in a dose-dependent manner (Figure I in the online-only Data Supplement).

To confirm that STAT3 activation occurred in cardiomyocytes, we performed immunohistological analyses to detect the nuclear localization of activated p-STAT3 (Figure 1C). Nuclear staining of p-STAT3 was detected in the IL-11–treated hearts but not in untreated hearts. Notably, >90% of nuclei of sarcomeric α-actinin–positive cells were also positively stained with anti-p-STAT3 antibody, indicating that IL-11 treatment results in STAT3 activation in cardiomyocytes in vivo. As is the case with noninfarcted mice, p-STAT3 was localized mainly in cardiomyocyte nuclei of postinfarct hearts, and IL-11 treatment increased the frequency of p-STAT3–positive cardiac myocytes (Figure II in

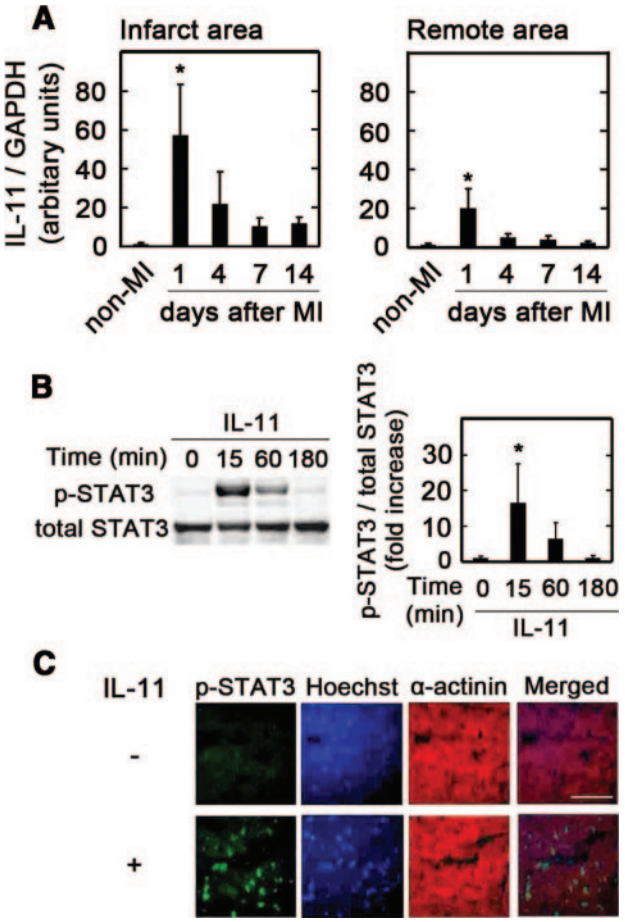

Figure 1. The transcript of IL-11 was elevated after MI, and IL-11 activated STAT3 in cardiomyocytes in murine hearts. A, Expression of IL-11 in infarct area or remote area of postinfarct hearts was analyzed by real-time RT-PCR. Expression of IL-11 was normalized with that of GAPDH. Data are shown as mean±SD (n=4 mice for each condition). *$P<0.05$ vs non-MI (sham) by 1-way ANOVA followed by Bonferroni test. B, IL-11 (8 μg/kg) was administered intravenously in mice for the indicated time. The lysates from hearts were immunoblotted with anti-p-STAT3 antibody. Blots were reprobed with anti-STAT3 antibody. Representative data are shown (left). Quantitative analyses of p-STAT3 are shown (right). Data are shown as mean±SD (n=4 mice for each condition). *$P<0.05$ vs 0 minutes by 1-way ANOVA followed by Bonferroni test. C, Fifteen minutes after injection of IL-11 (8 μg/kg) or PBS, frozen sections were prepared from the hearts. The sections were costained with anti-p-STAT3 and anti-sarcomeric α-actinin antibodies. Hoechst 33258 staining was also performed to identify the nuclei. The images are representative of 20 fields obtained from 4 mice. Bar=50 μm.

IL-11 Administration Attenuates Cardiac Remodeling After MI

To examine the effects of IL-11 on adverse cardiac remodeling, IL-11 was administered to the mice after MI operation, and cardiac fibrosis was histologically estimated at day 14 after MI (Figure 2). Both fibrotic circumference and fibrotic area were reduced by IL-11 in a dose-dependent manner (Figures 2B and C). Treatment of IL-11 at 8 μg/kg achieved a submaximal reduction in fibrotic circumference by 28.9% (PBS, 58.6±9.6%; IL-11, 41.7±10.0%) and fibrotic area by 33.1% (PBS, 39.8±9.3%; IL-11, 26.6±7.4%). Interestingly, IL-11–treated hearts showed an increase in infarct wall

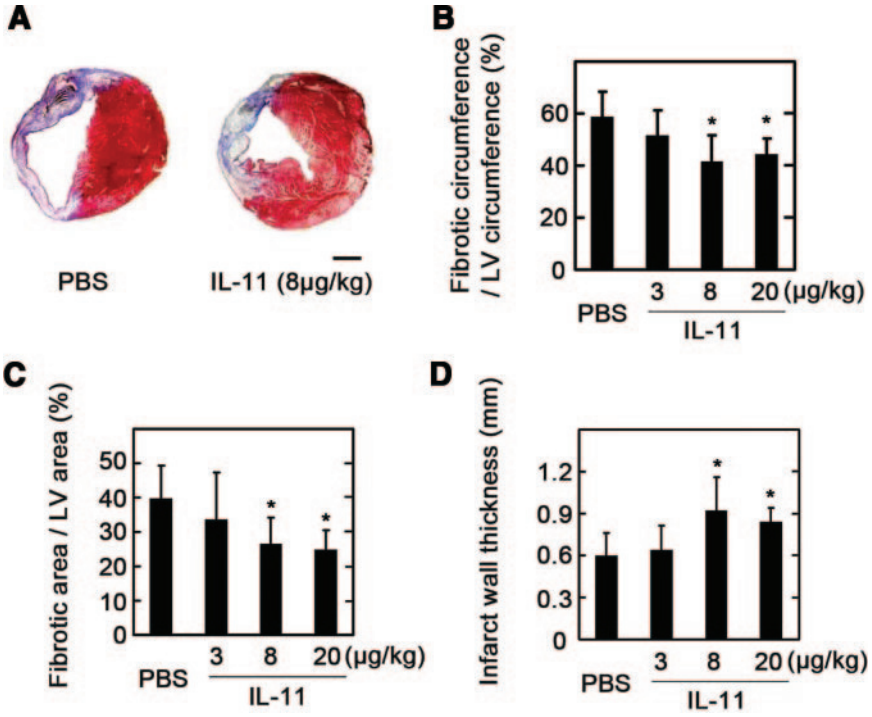

Figure 2. IL-11 attenuated cardiac fibrosis after MI. Heart sections (3 sections from each mouse) were prepared 14 days after MI and stained with Masson's trichrome. A, Images are representative of 24 to 27 obtained from 8 to 9 mice. Bar=1 mm. B to D, Ratio of fibrotic circumference to LV circumference (B), ratio of fibrotic area to LV area (C), and infarct wall thickness (D) were quantitatively estimated. Data are shown as mean±SD (n=8 mice for PBS; n=9 mice for 3 μg/kg; n=9 mice for 8 μg/kg; n=8 mice for 20 μg/kg). *$P<0.05$ vs PBS by 1-way ANOVA followed by Bonferroni test.

examine the effects of IL-11 on LV hypertrophy, we analyzed expression of α-skeletal muscle actin mRNA, a well-known marker of LV hypertrophy (Figure III in the online-only Data Supplement). IL-11 treatment showed a tendency to reduce α-skeletal muscle actin expression, although its reduction was not statistically significant.

To clarify whether IL-11 prevents cardiac dysfunction after MI, we measured LV developed pressure and ±dP/dt by a Langendorff apparatus. As acute myocardial damage, MI rapidly impaired LV developed pressure and ±dP/dt at day 1 (Table I in the online-only Data Supplement) before IL-11 administration was started. Because IL-11 treatment attenuated cardiac fibrosis that occurred during the following 2 weeks, we examined the effects of IL-11 on cardiac function 2 weeks after MI (Table). IL-11 treatment ameliorated chronic cardiac dysfunction in a dose-dependent manner compared with the PBS-treated group. Consistent with the attenuation of fibrosis, treatment of IL-11 at a dose of 8 μg/kg submaximally prevented cardiac dysfunction. Thus, further experiments were performed with the use of IL-11 at a dose of 8 μg/kg.

We also confirmed the inhibitory effects of IL-11 on adverse cardiac remodeling 28 days after MI. IL-11 treatment prevented cardiac fibrosis and preserved cardiac function (Figure IV in the online-only Data Supplement).

To examine whether IL-11 reduces infarct size, infarct size was measured by Evans blue exclusion 2 days after MI. There was no significant difference in infarct size (PBS, 23.0±7.1%; IL-11 [8 μg/kg], 22.0±4.3%; n=4 mice for each group).

Table. Effects of IL-11 on Cardiac Function at Day 14 After MI

		IL-11, μg/kg		
Parameters	PBS	3	8	20
LVDP, mm Hg	32.4±4.4	34.9±6.3	45.3±6.3*	44.3±13.5*
+dP/dt, mm Hg/s	884±113	958±162	1210±148*	1243±324*
−dP/dt, mm Hg/s	−713±93	−737±176	−1022±210*	−1043±326*

Data are mean±SD (n=8 mice for PBS; n=9 mice for 3 μg/kg; n=9 mice for 8 μg/kg; n=8 mice for 20 μg/kg). LVDP indicates LV developed pressure.

IL-11 Treatment Exhibits Antiapoptotic and Proangiogenic Activity in the Heart

Granulocyte colony-stimulating factor (G-CSF) was reported to prevent cardiac remodeling after MI, accompanied by antiapoptotic and proangiogenic effects through STAT3.[20] Because IL-11 activated STAT3 in cardiomyocytes (Figure 1), we examined the effects of IL-11 on cardiomyocyte survival and vascular formation in postinfarct myocardium.

To evaluate the antiapoptotic effects of IL-11, TUNEL staining was performed (Figure 3A). TUNEL-positive cardiomyocytes were detected mainly at the border zone, adjacent to the ischemic area, at both day 2 and day 4 after MI. Importantly, IL-11 treatment significantly reduced the number of apoptotic cardiomyocytes compared with the PBS group. To further confirm the cytoprotective effects, the cleavage of caspase 3 was examined by immunoblot analyses with cleaved caspase 3–specific antibody (Figure 3B). Compared with PBS treatment, IL-11 significantly reduced the band intensity for cleaved caspase 3. To address the antiapoptotic pathways downstream of the IL-11 signal, we examined the expression of cell survival proteins (Figure 3C). Immunoblot analysis revealed that Bcl-2 and survivin proteins, both of which have been characterized as downstream targets of STAT3,[21–23] were increased in the IL-11 group (Bcl-2, 1.9-fold; survivin, 1.5-fold).

Because activation of STAT3 mediated vascular formation

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