Toll-Like Receptor-2 Modulates Ventricular Remodeling After Myocardial Infarction

Tetsuro Shishido, MD; Naoki Nozaki, MD, PhD; Seiji Yamaguchi, MD, PhD; Yoko Shibata, MD, PhD; Joji Nitobe, MD, PhD; Takuya Miyamoto, MD; Hiroki Takahashi, MD; Takanori Arimoto, MD; Kunihiko Maeda, MD, PhD; Mitsunori Yamakawa, MD, PhD; Osamu Takeuchi, MD, PhD; Shizuo Akira, MD, PhD; Yasuchika Takeishi, MD, PhD; Isao Kubota, MD, PhD

Background—Toll-like receptors (TLRs) are members of the interleukin-1 receptor family and transduce similar signals as interleukin-1 receptor in response to exogenous pathogens. Recent studies have demonstrated that TLRs are activated by endogenous signals, such as heat shock proteins and oxidative stress, that may contribute to ventricular remodeling after myocardial infarction. In this study, we determined whether TLR-2 was involved in cardiac remodeling after myocardial infarction.

Methods and Results—Myocardial infarction was induced by surgical left anterior descending coronary artery ligation on wild-type (WT) mice and TLR-2–knockout (KO) mice. The survival rate was significantly higher in KO mice than in WT mice 4 weeks after myocardial infarction (65% versus 43%, P<0.03). Infarct size and degree of inflammatory cell infiltration in infarct area were similar between WT and KO mice. However, myocardial fibrosis in the noninfarct area of KO mice was much less than in WT mice (P<0.01) and was accompanied by reduced transforming growth factor-β1 and collagen type 1 mRNA expressions (P<0.01 and P<0.05, respectively). Left ventricular dimensions at end diastole were smaller in KO mice than in WT mice at 1 week (P<0.05) and 4 weeks (P<0.01) after surgery. Furthermore, fractional shortening was higher (27.7±2.5% versus 21.2±2.6%, P<0.05, at 1 week, and 24.3±2.0% versus 16.6±2.5%, P<0.01, at 4 weeks) in KO mice compared with WT mice.

Conclusions—These data suggest that TLR-2 plays an important role in ventricular remodeling after myocardial infarction. (Circulation. 2003;108:2905-2910.)

Key Words: receptors | remodeling | myocardial infarction | heart failure

Toll-like receptors (TLRs) recognize exogenous ligands, such as lipopolysaccharide, peptidoglycan, bacterial lipoprotein, and oligonucleotides, during the inflammatory responses.1,2 TLRs have interleukin-1 receptor–like intracellular signaling pathways that lead to nuclear localization of nuclear factor (NF)-κB/Rel-type transcription factors.1–3 Furthermore, TLRs are expressed in various organs, such as lung, brain, kidney, and heart.2–4 In vitro studies using isolated cardiomyocytes and in vivo studies using C3H/HeJ mice, which are TLR-4 deficient, have suggested that myocardial activated TLR-mediated signaling pathways in response to exogenous ligands induce cardiac dysfunction.5–7 Recent studies have demonstrated that TLR-mediated signaling pathways are also activated by endogenous ligands, such as heat-shock protein and oxidative stress, in isolated ventricular cardiomyocytes.8,9

A large myocardial infarction induces ventricular remodeling, which contributes to cardiac dysfunction and mortality.10,11 The ventricular remodeling process involves several mediators, such as oxidative stress,12 cytokines,13 and neurohormones.14 Several lines of evidence have suggested that oxidative stress alters cell survival and hypertrophic signals that lead to cardiomyocyte apoptosis and/or hypertrophy in isolated cardiomyocytes and in animals.12,15–18

Because oxidative stress after myocardial infarction was identified as playing a pivotal role for ventricular remodeling, we hypothesized that TLR-2 deficiency resulted in derangement of ventricular remodeling and preservation in cardiac function after myocardial infarction. To evaluate the potential role of TLR-2 on ventricular remodeling after myocardial infarction, we examined the effects of TLR-2 deficiency on cardiac function, structural aspects of ventricular remodeling,
and survival rates after myocardial infarction in a mouse model.

Methods

Animals
Male TLR-2–knockout (KO) mice and wild-type (WT) mice with the same C57BL/6 backgrounds were used. Mice were housed in a facility with a 12/12-hour light/dark cycle and were given free access to water and standard rodent chow. The room was kept specific pathogen–free. The animals were handled according to the animal welfare regulations of Yamagata University, and the study protocol was approved by the Animal Subjects Committee of Yamagata University. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Left Coronary Artery Ligation
Induction of myocardial infarction was performed as described previously,14,16 with minor modifications. Briefly, mice (25 to 35 g body weight) were anesthetized by intraperitoneal injection with a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg). Animals were intubated with a 20-gauge polyethylene catheter and were ventilated with a rodent ventilator (Harvard Apparatus). A median sternotomy was performed, the left anterior descending artery was identified, and a 7-0 proline suture was passed around the artery and subsequently tied off. Infarction was evident from discoloration of the left ventricle (LV). Finally, the chest wall was closed. The animals remained in a supervised setting until fully conscious.

Echocardiography
Transsthoracic echocardiography was performed as described previously14 with a FSonic 8900 (Fukuda Denshi) with a 10-MHz phased-array transducer at 1 or 4 weeks after surgery. LV internal dimensions at end systole and end diastole (LVEDD and LVESD) were measured digitally on the M-mode tracings and averaged from 3 cardiac cycles.14,16 LV fractional shortening (%FS) was calculated as \( \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100 \).

Morphological Examination and Infarct Size Measurement
At 3 days, 1 week, and 4 weeks after surgery, the heart was excised and fixed with a 10% solution of formalin in PBS. The heart was embedded in paraffin and cut serially from the apex to the base. The sections embedded in paraffin were incubated with primary antibody specific to activated macrophages (Mac-3 monoclonal antibody, BD Pharmingen) for 16 hours, followed by biotinylated secondary antibody (biotinylated anti-rat antibody, Jackson ImmunoResearch Laboratories, Inc) and then by avidin-conjugated horseradish peroxidase. Color development was performed with 3,3'-diaminobenzidine, and slides were counterstained with hematoxylin.19 The degree of macrophage emigration was expressed as a percentage of Mac-3–positive cells to total nucleated cells in infarct area.

Extraction of Total RNAs and Reverse Transcriptase–Polymerase Chain Reaction
Total RNAs were extracted from noninfarct LV using TRIzol (Invitrogen, Tokyo, Japan) and subjected to reverse transcriptase reverse transcriptase–polymerase chain reaction (RT-PCR) amplification using oligonucleotide primers.19 PCR primers for transforming growth factor-β1 (TGF-β1) were 5'-ACCCGAAACCACCCCTCTATG-3' (forward) and 5'-GTAACCCAGGAAATTGTCG-3' (reverse), primers for collagen type I were 5'-GTGAACTCCTGGCAAACCCG-3' (forward) and 5'-CTG-GAGACCAGAAGAGCACC-3' (reverse), and primers for GAPDH were 5'-ACTCCACTCTACGCGAATTCGCG-3' (forward) and 5'-AGGAGGGGAGATGATGACCC-3' (reverse).19 The PCR products were fractionated on a 1% to 2% agarose gel and visualized by ethidium bromide staining. The intensities of the bands were normalized for GAPDH and were expressed as fold increase over control WT.

Statistical Analysis
All values are expressed as mean±SEM. Comparisons of the histological analysis between WT and KO mice were made by Student’s unpaired t test. To compare body weight, LV weight, lung weight, LVEDD, LVESD, %FS, and RT-PCR data, 1-way ANOVA followed by post hoc test was performed. Survival curves after myocardial infarction were created by the Kaplan-Meier method and compared by log-rank test. Statistical significance was accepted at a value of \( P<0.05 \).

Results

Heart Failure and Ventricular Remodeling After Myocardial Infarction
There were no differences in body weight among 4 groups before and after myocardial infarction. Heart weight and LV weight after myocardial infarction were lower in KO mice than in WT mice (Table). Furthermore, lung weight was also lower in KO mice than in WT mice (Table).

Survival Rates and Infarct Size
The survival rates after myocardial infarction were compared up to 4 weeks between WT (n=107) and KO (n=80) mice, as shown in Figure 1. The rate of death during surgery was not different between WT and KO mice (14% and 15%, respectively). The survival rate up to 4 weeks after myocardial infarction was significantly higher in KO mice than in WT mice (65% versus 43%, \( P<0.03 \)). However, infarct size at 4 weeks after coronary ligation was similar between WT and KO mice, as shown in Figure 2 (55.6±4.2% and 53.2±3.7%, respectively).

Assessment of Inflammatory Cell Infiltration Into the Infarct Area
At 3 days and 1 week after myocardial infarction, histological sections of the infarcted area were evaluated to assess the degree of accumulated polymorphonuclear neutrophils and macrophages, because it has been reported that these are important sources for chemokine and cytokine induction or production.20 After myocardial infarction, polymorphonuclear neutrophils and activated Mac-3–positive macrophages were infiltrated into the infarct area in WT and KO mice (data not shown). However, morphometric analysis demonstrated
that there was no significant difference in the degree of inflammatory cell infiltration between WT and KO mice.

The numbers of polymorphonuclear neutrophil infiltration (per 10^4 mm^2) were 41.2±3.5 and 41.0±2.9 at 3 days after myocardial infarction and 54.3±2.8 and 55.0±3.3 at 1 week after myocardial infarction in WT and KO mice, respectively. The degrees of Mac-3–positive macrophage infiltration were 41.0±4.0% and 41.1±3.4% at 3 days and 45.1±4.0% and 44.2±3.3% at 1 week after myocardial infarction in WT and KO mice, respectively.

### Echocardiographic Measurements

Echocardiography was performed at baseline control and at 1 or 4 weeks after myocardial infarction in WT and KO mice. Under anesthesia, the heart rate was similar between WT and KO mice at each point (data not shown). The representative M-mode echocardiograms of WT and KO mice at baseline and 1 and 4 weeks after myocardial infarction are shown in Figure 3. Before coronary ligation, LVEDD, LVESD, and %FS were similar between WT and KO mice (baseline). LV dilatation after myocardial infarction was evident in both WT and KO mice; however, KO mice showed less ventricular dilatation. LVEDD and LVESD were smaller in KO mice than in WT mice at 1 and 4 weeks after operation, as shown in Figure 4, A and B. LVEDDs (mm) were 4.18±0.11 (n=13) and 4.61±0.17 (n=12) at 1 week (P<0.05) and 4.64±0.13 (n=15) and 5.13±0.16 (n=13) at 4 weeks (P<0.01) in KO mice and WT mice, respectively. LVESDs (mm) were 3.04±0.15 and 3.74±0.19 (P<0.05) at 1 week and 3.53±0.16 and 4.30±0.23 (P<0.01) at 4 weeks in KO mice and WT mice, respectively. Moreover, %FS was significantly higher at 1 and 4 weeks after surgery in KO mice than in WT mice, as shown in Figure 4C (27.7±2.5% versus 21.2±2.6%, P<0.05, at 1 week and 24.3±2.0% versus 16.6±2.5%, P<0.01, at 4 weeks). Both WT and KO mice showed linear relationships (r=0.68 and r=0.62, respectively, P<0.05) between infarct size and %FS at 4 weeks (Figure 4D).

### Assessment of Myocardial Fibrosis in Noninfarct Area

Morphological changes at the noninfarct area were similar between WT and KO mice at 3 days after myocardial infarction (data not shown). At 1 and 4 weeks after surgery, cardiomyocytes showed mild architectural alteration in KO mice compared with WT mice (data not shown). Myocyte cross-sectional area was not different between KO mice (331.2±48.8 μm^2) and WT mice (407.6±66.2 μm^2). In KO mice, the degrees of myocardial fibrosis at the noninfarcted interstitial area (0.34±0.06 versus 1.00±0.22, P<0.01) and perivascular area (0.35±0.04 versus 1.00±0.17, P<0.01) were much less than in WT mice at 4 weeks after myocardial infarction (Figure 5, A–E).
To investigate whether these morphological observations were accompanied by alterations in gene expression relevant to fibrotic changes, we measured mRNA levels of TGF-β and collagen type 1 at 1 week after myocardial infarction. RT-PCR revealed that TGF-β and collagen type 1 mRNA obtained from noninfarcted myocardium at 1 week after myocardial infarction in both WT and KO mice. However, these changes in TGF-β and collagen type 1 mRNA expressions after myocardial infarction were attenuated in KO mice compared with WT mice, as shown in Figure 6A. When normalized for GAPDH mRNA, the expressions of TGF-β and collagen type 1 in KO mice were reduced by 34.2% (P<0.01) and 38.6% (P<0.05), respectively, compared with WT mice (Figure 6B).

Discussion
In the present study, we examined whether ventricular remodeling after myocardial infarction was altered in TLR-2–KO mice. TLR-2–KO mice demonstrated higher survival rate and less ventricular remodeling evaluated by echocardiography, associated with fewer pathological changes and decreased expression of TGF-β and collagen type 1 mRNA. The present study provided the direct evidence for involvement of TLR-2–mediated signaling pathway in ventricular remodeling after myocardial infarction.

Previous studies have documented that TLRs are expressed in many organs, such as spleen, brain, kidney, liver, lung, and heart, and act differentially in a wider pattern of tissue expression. In an in vivo study, Nemoto et al. demonstrated that myocardial TLRs are involved in cardiac dysfunc-
tion during inflammatory shock. However, the potential role of TLR-2 in ventricular remodeling after myocardial infarction is still unknown. In this study, at 1 and 4 weeks after myocardial infarction, LVEDD and LVESD were smaller in TLR-2–KO mice than in WT mice. Furthermore, %FS was markedly higher in TLR-2–KO mice than WT mice, which was associated with a better survival rate in KO mice. These results suggest that TLR-2 plays an important role in ventricular remodeling after myocardial infarction.

Several studies have demonstrated that myocardial infarction is associated with inflammatory responses, which can extend myocardial injury. Reduced numbers of neutrophils by antineutrophil drugs or neutrophil filters can reduce myocardial injury after ischemia. Furthermore, Li et al demonstrated that necrotic cells activate NF-kB in macrophages and induce expression of genes involved in inflammatory response through TLR-2–mediated signaling pathways. In our present study, activated macrophages stained with anti-Mac-3 antibody and polymorphonuclear neutrophil accumulation infiltrated into the infarct area were unexpectedly similar between WT and TLR-2–KO mice at 3 days and 1 week after myocardial infarction. However, we did not exclude the possibility for attenuated activity of inflammatory cells in TLR-2–KO mice.

TLR-2 is known to recognize exogenous ligands in response to such pathogens as oligonucleotides and bacterial lipoprotein from gram-positive bacteria. Although several studies have demonstrated that neurohormones modulate ventricular remodeling, TLR-2 has an interleukin-1 receptor–like intracellular signaling domain, and there is no evidence that TLR-mediated signaling pathways are activated by such factors. It has been demonstrated that before injection of lipopolysaccharide, hemodynamic and echocardiographic parameters are similar between TLR–deficient and WT mice. In the present study, there was no difference in cardiac function at baseline between WT and KO mice.

Accumulating evidence has reported that oxidative stress contributes to ventricular remodeling after myocardial infarction. Frantz et al demonstrated that endogenous ligands, such as oxidative stress, upregulate NF-kB DNA binding activity through the TLR–mediated signaling pathway in cardiomyocytes and Chinese hamster ovary fibroblasts. In this study, cardiac dysfunction evaluated by echocardiography at 4 weeks after myocardial infarction was abrogated in TLR–KO mice compared with WT mice. Therefore, ventricular remodeling after myocardial infarction was modulated through TLR-2.

In this study, histological examinations demonstrated that myocardial fibrosis was decreased significantly in KO mice at noninfarct area. Furthermore, gene expressions of TGF-β, and collagen type 1, which were responsible for cardiac fibrosis, were lower in KO mice than WT mice after myocardial infarction.
myocardial infarction. These data support the concept that modulation of myocardial fibrosis in the noninfarct area is one of the possible mechanisms for attenuation of ventricular remodeling in TLR-2–KO mice.

We reported the preservation of cardiac function, increased survival rate, and attenuation of myocardial fibrosis after myocardial infarction in TLR-2–KO mice. In conclusion, this study demonstrates the first evidence that ventricular remodeling after myocardial infarction occurs, at least in part, through the TLR-2–mediated pathway. These results will allow us a novel approach to investigate the pathogenesis of ventricular remodeling after myocardial infarction.

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