Activation of TGF-β1-TAK1-p38 MAPK pathway in spared cardiomyocytes is involved in left ventricular remodeling after myocardial infarction in rats

Madoka Matsumoto-Ida,* Yoshihito Takimoto,* Takeshi Aoyama, Masaharu Akao, Toshihiro Takeda, and Toru Kita

Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

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C O N T E N T S

MATERIALS AND METHODS

Animals with MI. We performed animal experiments in accordance with the Declaration of Helsinki, and these were approved by our institutional ethics committee for animal experiments.

MI was surgically induced in 8-wk-old male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) weighing 280–320 g by ligation of the left anterior descending coronary artery, as described previously (1, 26). In brief, the chest was opened via an anterior thoracotomy, and the heart was rapidly exteriorized. The proximal left anterior descending coronary artery was ligated about 2 mm distal to its aortic origin with a 7-0 silk suture. Successful ligation was confirmed by regional cyanosis of the myocardial surface. The heart was returned to its original position, and the incision was closed. Sham-operated animals underwent identical surgery except for the coronary artery ligation. After the operation, rats were allowed free access to standard rat chow, and water was provided ad libitum.

The overall mortality of MI rats during the entire experimental period (up to 14 days after MI) was 30–40%. The majority of death occurred on the day or the following day of the MI surgery, probably

CARDIAC REMODELING refers to the process by which the normal cardiac architecture is altered in response to myocardial infarction (MI) (4, 16, 23, 24). Although remodeling is an adaptive mechanism against increased wall stresses, it also contributes to the progression of heart failure; decreased ventricular compliance contributes to diastolic dysfunction, which over time is followed by the development of progressive contractile dysfunction. Microscopically, remodeling is featured by hypertrophic growth of cardiomyocytes and hyperplastic growth of cardiac fibroblasts, as well as wound healing in the scar. These histological changes are based on various alterations of genetic expression: the reexpression of a “fetal” gene program, including the upregulation of genes such as β-myosin heavy chain (β-MHC) and atrial natriuretic peptide (ANP) (19, 22).

Inflammatory cytokines play pivotal roles in post-MI left ventricular remodeling (12). Among them, transforming growth factor (TGF)-β1 is upregulated after MI (3, 27, 29) and constitutes the signaling network with the renin-angiotensin system to promote cardiac remodeling (17). TGF-β1 causes the induction of fetal gene expression in cardiomyocytes and stimulates the production of extracellular matrix protein by cardiac fibroblasts (6, 15, 17). TGF-β1 elicits its biological responses through a heteromeric receptor complex comprising two serine-threonine kinase receptors, termed TGF-β receptor types I and II (TβRI and TβRII) (10). The signal is transduced to a member of the MAPK kinase kinase (MAPKKK) family, TGF-β-activated kinase (TAK1), which subsequently activates MKK3/6 (MAPKK) and p38 MAPK (30). Zhang et al. (32) reported that an activating mutation of TAK1 expressed in myocardium of transgenic mice produced p38 MAPK phosphorylation in vivo, cardiac hypertrophy, interstitial fibrosis, severe myocardial dysfunction, “fetal” gene induction, apoptosis, and early lethality. However, the role of TAK1 in post-MI remodeling remains to be determined.

Therefore we hypothesized that the TGF-β1-TAK1-MKK3/6-p38 MAPK pathway may be activated during ventricular remodeling after MI. We examined the protein levels of these peptides, their localizations, and the correlation between this pathway and ventricular remodeling, using a rat model of MI.

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due to acute pump failure or lethal arrhythmias. After MI was produced, rats were randomly selected for death at various time points.

**Determination of infarct size.** The rats were anesthetized with pentobarbital, and their hearts were arrested with potassium chloride and rapidly excised. After body weight and heart weight were measured, three thin transverse slices were cut from the apex to base, and these were embedded with an OCT compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. The infarct size was determined using the triphenyltetrazolium chloride (TTC) staining method as described previously (1, 26). Rats with infarcts occupying <35% of the left ventricle were excluded from the analysis because less significant cardiac remodeling was expected in rats with small-sized MI. Roughly 10% of animals were excluded based on this criterion.

**Echocardiographic studies.** To evaluate the left ventricular dimensions and function, transthoracic echocardiography (Hewlett-Packard, Palo Alto, CA) was performed at each time point with a 7.5-MHz sector scan probe. M-mode echocardiograms at the papillary muscle level were recorded using two-dimensional long-axis images as a guide under mild anesthesia with pentobarbital (15 mg/kg im). The left ventricular end-diastolic and end-systolic dimensions were measured from the M-mode tracing. The left ventricular fractional shortening was calculated as described previously (1, 26). The systolic blood pressure of each animal was measured before echocardiographic studies by the tail-cuff method.

**Western blot analysis.** For all Western blot analyses, noninfarct myocardial tissue samples were obtained. Lysates (60 μg) from heart tissues, or the immunoprecipitates, were separated by a 10% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were immunoblotted with anti-TGF-β1, -TBR1, -TBR2, -TAK1, -MKK3/6, -phosphorylated MKK3/6 (P-MKK3/6), -p38 MAPK, or -phosphorylated p38 MAPK (P-p38) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

**In vitro kinase assay.** The amount of activated form of TAK1 was measured by in vitro kinase assay (11); lysates from noninfarct myocardium were immunoprecipitated by anti-TAK1 antibody, and the immunoprecipitates were then incubated with recombinant MKK3/6 (Upstate Biotechnology, Lake Placid, NY) (20 μg/ml) and 100 μM γ-[32P]ATP (1 μC) in a solution containing 20 mM Tris-Cl, pH 7.5, 2 mM EGTA, and 10 mM MgCl2 for 20 min at 30°C. The reaction was stopped by boiling the samples. After SDS-polyacrylamide gel electrophoresis, phosphorylation of MKK3/6 was detected by autoradiography.

Immunoprecipitation was carried out as follows: noninfarct myocardial tissue samples were homogenized with a tissue homogenizer in a lysis buffer (50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.25% sodium deoxycholate; 1 mM EDTA; 2 μM leupeptin; 1 μM PMSF). The debris was removed by centrifugation at 1,000 g for 15 min. The protein concentration of the lysates was determined with the Bradford protein assay (Bio-Rad, Hercules, CA). Lysates were incubated with an anti-TAK1 antibody (Santa Cruz Biotechnology) and protein A-agarose (Amersham) for 3 h at 24°C. The immunoprecipitates were washed three times and eluted with 20 μl of sample buffer (62.5 mM Tris, pH 6.8; 2% SDS; 20 mM DTT; and 1% glycerol).

**Real-time RT-PCR.** For analysis of mRNA levels for TAK1, TBR1, TBR2, ANP, and β-MHC, total RNA was isolated from myocardial tissue samples by use of the RNeasy Mini Kit (Qiagen, Valencia, CA). Subsequently, DNase-treated total RNA was reverse-transcribed by use of SuperScriptIII First Strand Synthesis System (Invitrogen, Carlsbad, CA). Measurements of mRNA levels were performed by real-time reverse transcription-polymerase chain reaction (RT-PCR) by use of an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). A 25-μl reaction mixture was used that contained 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), 10 ng of cDNA template, and the following primers: 5′-CGCCATCGAGGCTGTTAAAC-3′ (sense), 5′-GGCTGCACTGCTGCAAGTGTA-3′ (antisense) for TAK1; 5′-GGACTTGGTCTGGAGACATAGA-3′ (sense), 5′-CATGGCTCCATGGCGCAT3′- (antisense) for TβRI; 5′-TTCACCTAAGCAGGCTC-3′ (antisense), 5′-CAGGATAGTGGCCAGGTTGTG-3′ (antisense) for TβRII; 5′-TATACAGTGCCGGTGCAAC-3′ (sense), 5′-GCTCCATACCTG-GCACTC-3′ (antisense) for ANP; 5′-GAAGGGATGAGTGCACAA-CATG-3′ (sense), 5′-AGCTGAGCACTGCTTCTG-3′ (antisense) for β-MHC; and 5′-ACACAGTCCATGGCATTAC-3′ (sense), 5′-TCCACACCTTGGTGCAGTA-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In each experiment, a standard curve for each primer pair was obtained using a serial dilution of a recombinant plasmid containing cDNA. The threshold cycle (Ct) was subsequently determined. Relative mRNA levels were calculated based on the Ct values and normalized by the GAPDH level of each sample. To avoid the possibility of amplifying contaminating DNA, all the primers for real-time RT-PCR were designed with an intron sequence inside the cDNA to be amplified, and reactions were performed with appropriate negative control samples (template-free control samples).

**Immunohistochemistry.** The avidin-biotin peroxidase complex technique was used. Five-micrometer-thick sections were cut from heart tissues surrounded with the OCT compound and fixed with 2% paraformaldehyde. Intrinsic peroxidase activity was blocked with 0.25% H2O2. The sections were blocked with 5% bovine serum albumin and then incubated at 4°C with the anti-TβRI, -TβRII, or -TAK1 antibody overnight. After a wash with TTBS (0.1% Tween 20, 0.9% NaCl in 0.1 M Tris-Cl, pH 7.4), a biotinylated anti-rabbit or mouse IgG antibody was added, and incubation was carried out with a streptavidin-peroxidase complex (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected by using 3,3′-diaminobenzidine, and slides were then counterstained with hematoxylin.

**Statistics.** Values are presented as means ± SE. The measurements from rats with MI were compared with those of sham-operated rats by using Student’s unpaired t-test. The significance among the means of various groups was analyzed by one-way ANOVA with post hoc comparisons by using the Tukey-Kramer test. Significance was taken as P < 0.05.

**RESULTS**

**Body and heart weights and infarct size.** Table 1 shows body and heart weight and infarct size of animals after the sham operation and after 1, 3, 7, and 14 days of MI. The heart weight and the heart-to-body weight ratio of the MI group 3 days postinfarct, by which time inflammation and edema had developed within the infarct, significantly increased (P < 0.05) compared with those of the sham-operated control group and then returned to the control values 7 days postinfarct and thereafter. All animals developed large MIs, with infarct size (determined by TTC staining) ranging from 37.7% to 52.8% of the left ventricle, and these values did not change significantly over the experimental period (by 14 days postinfarct).

**Hemodynamics and echocardiographic study.** Rats with MI exhibited progressive left ventricular remodeling, both hemo- dynamically and geometrically. Table 2 represents the hemodynamic parameters and echocardiographic data in sham-operated rats and those after 1, 3, 7, and 14 days of MI. In all of the animals with MI compared with the sham-operated animals, blood pressure was significantly lower, and heart rate was significantly higher at all time points. Left ventricular diastolic and systolic dimensions increased rapidly during the acute phase of MI, and progressive changes
were observed by 2 wk (P < 0.01 at each time point compared with sham-operated rats). In parallel, fractional shortening decreased rapidly during the acute phase due to akinesis of the anterior wall, and this systolic dysfunction persisted thereafter (P < 0.01 at each time point compared with sham-operated rats). Posterior wall thickness was significantly (P < 0.01) increased in rats with MI compared with sham-operated rats on day 3 and thereafter, which is a manifestation of compensated hypertrophy of noninfarct myocardium.

Western blotting of TGF-β1, TβRI, TβRII, TAK1, MKK3/6, P-MKK3/6, p38 MAPK, and P-p38 MAPK. Protein levels of components of the TGF-β1-TAK1-p38 MAPK pathway were determined by using Western blotting. TGF-β1 protein levels were increased compared with those in sham-operated rats by 1.30 ± 0.01-, 2.59 ± 0.10-, and 2.34 ± 0.06-fold, and 1.57 ± 0.03-fold (P < 0.01) at 1, 3, 7, and 14 days after MI, respectively (Fig. 1, A and B). TβRI and TβRII expression showed modest but significant upregulation, peaking at 3 days after MI (Fig. 1, A, C, and D).

In parallel with the upregulation of TGF-β1, the protein levels of TAK1 were significantly increased 1.66 ± 0.14-, 1.97 ± 0.16-, and 1.57 ± 0.07-fold (P < 0.01) at 1, 3, and 7 days post-MI compared with sham-operated rats (Fig. 2, A and B). Although there was no change in the protein levels of MKK3/6 and p38 MAPK levels throughout the time course, P-MKK3/6 and P-p38 MAPK were increased significantly (P-MKK3/6: 2.20 ± 0.19-, 2.55 ± 0.47-, and 3.29 ± 0.32-fold; P-p38 MAPK: 1.90 ± 0.03-, 2.03 ± 0.06-, and 2.35 ± 0.06-fold at 1, 3, and 7 days after MI), indicating the activation of the TGF-β1-TAK1-MKK3/6-p38 MAPK pathway (Fig. 2, A and B).
A, C, and D). At 14 days postinfarct, all of these returned to the control level (Fig. 2, A–D).

In vitro kinase assay. To directly demonstrate that TAK1 is activated in post-MI remodeling and is capable of activating the downstream substrates MKK3/6-p38 MAPK, we performed an in vitro kinase assay. Recombinant MKK3/6 was coincubated with heart protein samples immunoprecipitated by TAK1 antibody. As shown in Fig. 2E, phosphorylation of MKK3/6 was increased in animals with MI at 3 days postinfarct, a time when the TAK1 protein level reached a maximum.
Immunohistochemistry. We examined the localization of TβRI, TβRII, and TAK1 using immunohistochemical staining. Immunoreactive staining of TβRI (Fig. 3A) and TβRII (Fig. 3B) was observed in cardiomyocytes (black arrow), vascular smooth muscle cells (white arrowhead), and fibroblasts (black arrowhead) in the border zone of MI. In contrast, immunoreactivity for TAK1 was mainly localized to spared cardiomyocytes in the border zone of MI (Fig. 3C) and in the noninfarcted region (Fig. 3D), suggesting that the TGF-β1-TAK1 pathway was activated in cardiomyocytes but not in vascular smooth muscle cells or interstitial cells. In addition, TAK1 protein was not detected by Western blotting in the center of the infarcted region where there were no cardiomyocytes (data not shown).

mRNA levels of TAK1, TβRI, TβRII, ANP, and β-MHC. To determine whether the increases in TAK1 signaling occur at transcriptional level, we measured mRNA levels of TAK1.
TβRI, and TβRII, using real-time RT-PCR (Fig. 4, A and B). It was previously reported that TGF-β1 mRNA is upregulated after MI (27, 29). TAK1 mRNA levels were increased by 1.91 ± 0.22-, 1.65 ± 0.12-, 2.30 ± 0.19-, and 2.33 ± 0.24-fold at 1, 3, 7, and 14 days after MI, respectively (Fig. 4B), compared with those in sham-operated rats. mRNA levels of TβRI and TβRII at 3 days after MI were 5.50- and 2.37-fold, respectively. Taken together, the increases in the signaling pathway were due to transcriptional upregulation.

To determine whether the expression of fetal cardiac genes was increased in the ventricles of rats after MI, we measured mRNA levels of ANP (Fig. 4C) and β-MHC (Fig. 4D) by real-time RT-PCR. Both ANP and β-MHC mRNA levels were increased compared with sham-operated rats at all time points (Fig. 4, C and D): 12.6 ± 3.9-, 14.0 ± 2.1-, 20.5 ± 6.9-, and 8.5 ± 1.0-fold for ANP, and 3.4 ± 0.3-, 8.7 ± 0.9-, 2.4 ± 0.1-, and 1.9 ± 0.3-fold for β-MHC at 1, 3, 7, and 14 days after MI, respectively. Thus profound upregulation of ANP and β-MHC mRNA levels was observed in the spared myocardium, indicating the presence of hypertrophic remodeling process in our MI model.

DISCUSSION

Here we demonstrated that the protein levels of TGF-β1, TβRI, TβRII, and TAK1 were significantly increased in the noninfarcted myocardium in rats with MI compared with sham-operated animals. Phosphorylation of MKK3/6 and of p38 MAPK was also increased in the noninfarcted region. This signaling cascade appeared to reach a maximum at days 3–7 postinfarct and return to the control level by day 14. Moreover, activated TAK1 in noninfarcted myocardium was capable of activating recombinant MKK3/6, suggesting a causative role for TAK1 in the remodeling process. The immunoreactivity for TAK1 was mainly localized to cardiomyocytes, whereas that for TβRI and TβRII was observed in vascular smooth muscle cells and fibroblasts as well as cardiomyocytes. These lines of evidence suggest that the TGF-β1-TAK1-p38 MAPK pathway in cardiomyocytes of the noninfarcted region is activated during acute MI. In accordance with the activation of this pathway, ANP and β-MHC expression was increased. Although many trophic factors induce the expression of these genes, we clarified that TGF-β1 is one of the stimulators of cardiomyocyte hypertrophy during ventricular remodeling after acute MI and that this signal is transmitted through the TAK1-MKK3/6-p38 MAPK pathway.

Role of TGF-β1 in left ventricular hypertrophy and post-MI remodeling. TGF-β1 is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems (2). Studies with experimental models of MI and with pressure overload have shown increased myocardial TGF-β1 expression, suggesting the involvement of TGF-β1 in cardiomyocyte hypertrophy as well as fibrosis (3, 9, 25, 27, 29). Overexpression of TGF-β1 in transgenic mice results in interstitial fibrosis and hypertrophic growth of cardiomyocytes (18). TGF-β1 provokes the genetic upregulation of fetal contractile proteins, such as β-MHC and α-skeletal actin in cultured neonatal cardiomyocytes (15). However, the intracellular signaling pathway of TGF-β1-induced cardiomyocyte hypertrophy after MI has remained unknown. It has been demonstrated that activated MKK3/6-p38 MAPK induces the expression of genes encoding sarcomeric proteins and elicits sarcomeric organization in cultured cardiomyocytes (28, 31). In this study, we demonstrated that expression of TAK1, the stimulator of MKK3/6 in the signal transduction of TGF-β1, increased and the protein was localized to cardiomyocytes during acute MI.

TGF-β1 induced myocyte hypertrophy and collagen deposition. TGF-β1 induces extracellular matrix production in fibroblasts and promotes fibrosis. Two major signaling pathways, Smad proteins and TAK1, have been identified in the signal transduction of TGF-β1 in other types of cells (5, 8, 30). Smad signaling is particularly involved in collagen deposition in the extracellular matrix (17, 21). Indeed, the Smad pathway was also reported to be upregulated after MI in rats (7). Smad proteins were located mainly in the perivascular space in the noninfarcted region and in nonmyocyte cells, probably myofibroblasts, in the infarct scar. Although the role of Smad was not examined in these studies, Smad signaling seemed to play a major role in the elevated production of the extracellular matrix proteins in fibroblasts after MI. Although TGF-β1 was activated in both cardiomyocytes and fibroblasts after MI, the intracellular signaling pathways in these two distinct types of cells were different from each other. TAK1-p38 MAPK is activated in cardiomyocytes to induce myocyte hypertrophy.

Role of TAK1 in post-MI remodeling. Zhang et al. (32) reported that TAK1 is activated in cardiomyocytes after pressure overload. In transgenic mice with activated TAK1, considerable ventricular hypertrophy developed and led to impaired systolic and diastolic function. In this model, β-MHC mRNA expression was increased 20-fold compared with control mice. Comparable levels of β-MHC mRNA upregulation were observed in our rat MI model. The endogenous TAK1 may help to regulate β-MHC expression to prevent increased wall stress after MI. Although TAK1 was originally isolated as a target of TGF-β1 (30), TNF-α or IL-1 recently was also shown to activate TAK1 (13, 20). Given that TNF-α and IL-1 expression in the left ventricle is increased after MI (14), TNF-α or IL-1 may stimulate TAK1 in the cardiomyocytes during acute MI, although we did not determine the expression levels of these cytokines.

Pathophysiological implications. In conclusion, activation of the TGF-β1-TAK1-p38 MAPK pathway is at least one of the important responses to cardiac remodeling that is involved in the progression of heart failure. These observations suggest that this signaling pathway could be a novel therapeutic target for the suppression of post-MI remodeling. However, further studies are required to see whether the blockade of the TAK1-signaling pathway would lead to the inhibition of post-MI remodeling and subsequent heart failure.

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Present address of T. Aoyama: Kansai Electric Power Hospital, 2-1-7 Fukushima, Fukushima-ku, Osaka 553-0003, Japan.

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