Smad1 Protects Cardiomyocytes From Ischemia-Reperfusion Injury

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Background—We previously reported that bone morphogenetic protein 2 (BMP2) protected against apoptosis of serum-deprived cardiomyocytes via induction of Bcl-xL through the Smad1 pathway. To investigate whether Smad1 signaling promotes cell survival in the adult heart, we subjected transgenic mice with cardiac-specific overexpression of smad1 gene (Smad1TG) to ischemia-reperfusion (I/R) injury.

Methods and Results—The effects of BMP2 or adenovirus-mediated transfection of smad1 on cardiomyocyte survival in hypoxia-reoxygenation were examined using rat neonatal cardiomyocytes. BMP2 and Smad1 each significantly promoted survival and diminished apoptotic death of cardiomyocytes during hypoxia-reoxygenation. Interestingly, Smad1 was found to be activated during I/R in normal mouse heart. To examine physiological and pathological roles of Smad1 in I/R, we generated Smad1TG using the α-myosin heavy chain gene promoter. Phosphorylation of Smad1 was found in all smad1 transgene–positive mouse hearts. To examine whether Smad1 prevents injury of cardiomyocytes in vivo, we subjected Smad1TG and age-matched wild-type mice (WT) to I/R injury induced by 1 hour of ligation of the left coronary artery and 1 hour of reperfusion. TUNEL and DNA ladder analyses showed that Smad1TG had significantly smaller myocardial infarctions and fewer apoptotic deaths of cardiomyocytes than did WT. Interestingly, increased expression of Bcl-xL and β-catenin was more remarkable whereas caspase3 was less activated in Smad1TG heart than in that of WT.

Conclusions—These findings suggest that the Smad1 signaling pathway plays a role in cardioprotection against I/R injury. (Circulation. 2005;111:2752-2759.)

Key Words: apoptosis ■ hypoxia ■ ischemia ■ reperfusion ■ signal transduction

The recent finding that apoptosis of cardiomyocytes is characteristic of several myocardial diseases, including ischemic heart disease and congestive heart failure, has raised the hope that inhibition of myocyte apoptosis could prevent the loss of contractile cells and thus provide a new target in a multimodal therapeutic approach to cardiac disease.1

The importance of apoptosis in cell death after ischemia and reperfusion has been demonstrated in in vivo rodent models.2,3 In rats, ischemia-induced apoptosis and subsequent necrosis have been reported.4 There is more supportive evidence for ischemia-related apoptosis; mitochondrial dysfunction has been demonstrated with leakage of cytochrome c and caspase activation after global ischemia in the isolated rat heart. Borutaite et al5 showed that degree of caspase activation was dependent on time of ischemia. The release of cytochrome c after mitochondrial permeability transition activation appears to induce apoptosis.

We previously reported that bone morphogenetic protein 2 (BMP2) protected against apoptosis of serum-deprived cardiomyocytes via induction of Bcl-xL through the Smad1 pathway.6 By binding to 2 different types (I and II) of serine-threonine kinase receptors, BMP2 phosphorylates Smad1, thereby forming hetero-oligomeric complexes with Smad4, translocates into the nucleus, and modulates the transcription of a variety of target genes.7 With regard to the functional role of Smad1, targeted disruption of the smad1 gene in mice results in embryonic lethality at approximately day 10.5 of gestation, suggesting that Smad1 plays a critical role in early development.
role in early embryogenesis. Interestingly, immunohistochemical staining of Smad1 protein is stronger in adult than in embryonic heart. However, the roles played by Smad1 in cardiac pathophysiology have not been fully studied.

To elucidate the function of Smad1 in the adult heart, we generated mice with cardiac-specific overexpression of smad1 gene (Smad1TG) and examined whether Smad1 signaling promotes survival of cardiomyocytes in vivo. In the present study, we demonstrated that Smad1 protects cardiomyocytes from ischemia-reperfusion (I/R) injury. This protective effect was accompanied by a marked reduction in the proportion of apoptotic cardiac death. We also showed that the survival effect of Smad1 involved increased expression of Bcl-xL and β-catenin in the heart. Together with an in vitro examination showing that BMP2 protected against hypoxia-reoxygenation (H/R)-induced apoptosis of cardiomyocytes through the Smad1 pathway, these findings suggest that the BMP2/Smad1 signaling system plays an important role in cardioprotection and that BMP2/Smad1 may function as an attenuator of apoptosis in cardiomyocytes, a finding with potential therapeutic and prognostic implications.

**Methods**

**Cell Culture, H/R, and Generation of Recombinant Adenovirus**

Primary cultures of neonatal rat cardiomyocytes were prepared from the ventricles of 1-day-old Wistar rats (Kiwamoto, Watanabe, Japan) and cultured at a cell density 3 × 10^6/mL in 6-well dishes. The cells were exposed to hypoxia induced with an Anaero Pack (Mitsubishi Gas Chemical) for 24 hours, followed by reoxygenation for 5 hours, as previously described. Pretreatment with BMP2 (Yamanouchi Co, Ltd) was performed for 12 hours before hypoxia. The recombinant replication-defective adenovirus expressing Smad1 was prepared as described previously. Cardiomyocytes were infected with Smad1 adenoviral vectors (Ad-Smad1) at a multiplicity of infection of 20 for 12 hours. After removal of the viral suspension, cardiomyocytes were serum starved for 12 hours and subjected to H/R. Adenovirus vector expressing β-galactosidase (Ad-β-gal) was used as a control for Ad-Smad1. Cell viability was quantified by MTS assay (Promega).

**Smad1 Morpholino Antisense Oligonucleotide**

The antisense oligonucleotide for smad1 was a 25-nucleotide morpholino oligo (Gene Tools, LLC) with the base composition 5′-CAAGCTGGTACATCATGGCGCT-3′. A standard morpholino oligo with the base composition 5′-CTCTTACCTCAgTTAg-TTACAATTTATA-3′ was used as a control. Endo-porter (Gene Tools, LLC) was used to deliver morpholino oligos into cytosol of cardiomyocytes.

**Evaluation of Apoptotic Cell Death**

Apoptotic death of cardiomyocytes was determined by TUNEL and DNA ladder analysis, as previously described (for details, see the online-only Data Supplement).

**Assessment of Mitochondrial Membrane Potential**

Loss of mitochondrial membrane potential was assessed with a laser scanning confocal microscope (Nikon TE2000U microscope and Bio-Rad Radiance 2100 scanning system) after staining with 5,5′,6′,6-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanide iodine (JC-1, Molecule Probes). Cardiomyocytes were incubated with 2 μg/mL JC-1 for 10 minutes at 37°C. After the dye was applied, confocal images were obtained within 30 minutes with the confocal microscope. A 488-nm argon laser and 543-nm helium neon laser were used for excitation, and the resultant red and green fluorescences were quantified with LP 570-nm and BP 500- to 530-nm filters, respectively.

**Antisense Assay**

Bcl-xL antisense oligonucleotide assays were performed according to previously described methods.

**Generation of Smad1TG**

A 1.4-kb human smad1 cDNA, generously provided by Dr K. Miyazono (University of Tokyo, Tokyo, Japan), was ligated with a 5.5-kb DNA fragment containing the murine cardiac α-myosin heavy chain gene promoter. Transgenic mice were generated as described elsewhere. In all experiments, nontransgenic (wild-type [WT]) littermates were used as controls. The care of all animals used in the present study was in accordance with the Osaka University animal care guidelines.

**Culture of Cardiomyocytes From Smad1TG**

Cells were prepared from neonatal hearts of Smad1TG and seeded at a density of 1 × 10^6 cells per 0.1 mL per well. Cells were exposed to H/R, and cell viability was quantified by MTS assay.

**Western Blot Analysis**

Western blot analysis was performed as described previously. After blocking, the membranes were probed with anti-Smad1, –phospho-Smad1, –α-tubulin, –Bcl-xL, –Bcl-2, –Bax, –Bad, –caspase3, –caspase8, –cyclin-dependent kinase-4 (Cdk-4) (Santa Cruz Biotechnology, Inc), –β-catenin (Sigma), and –cytochrome c (BD Biosciences Pharmingen) antibodies. The ECL system (Amersham Biosciences, Inc) was used for detection. Cytochrome c, Bax, and caspase3 were quantified in cytosol and mitochondrial fractions prepared with the Mitochondria/Cytosol Fractionation Kit (Bio Vision).

**Northern Blot Analysis**

Northern blot analysis was performed as described previously. The probe for GAPDH was kindly donated by Dr K.R. Chien (University of California, San Diego). Murine bcl-xL cDNA and murine bcl-2 cDNA, kindly donated by Dr Y. Tsujimoto (Osaka Graduate School of Medicine, Osaka, Japan), were used as probes.

**In Vivo I/R and Assessment of Myocardial Infarction**

I/R was produced in Smad1TG and WT (10- to 12-week-old males in each group) by transiently ligating the left coronary artery. The mice underwent 1 hour of occlusion, followed by 1 hour of reperfusion. Infarct sizing was performed as described in a previous study.

**Immunohistochemistry and Image Analysis**

At the end of 1 hour of reperfusion after 1 hour of occlusion, the hearts were extracted, frozen, and sectioned perpendicular to the long axis. Sections of Smad1TG and WT were analyzed for reactivity with antibody against β-catenin. Antigen-antibody complexes were visualized with 3,3-diaminobenzidine (DAB). To confirm accumulation of β-catenin protein, quantitative analysis was performed with a computerized image analysis system.

**Statistical Analysis**

Values are shown as mean±SD. Multiple-group comparison was performed by 1-way ANOVA, followed by the Bonferroni procedure for comparison of means. A 2-tailed Student t test was used to compare Smad1TG with WT specimens under identical conditions. Values of P<0.05 were considered significant.

**Results**

BMP2/Smad1 Promotes Survival and Inhibits Apoptosis During H/R in Cultured Cardiomyocytes

In cultured cardiomyocytes pretreated with BMP2, Smad1 was transiently phosphorylated 15 minutes after BMP2 stim-
ulation and again phosphorylated on reoxygenation (Figure 1A and 1B). Rephosphorylation during reoxygenation was attenuated in cardiomyocytes after pretreatment of BMP2, followed by placement in BMP2-free medium just before H/R (Figure 1A and 1B). These data suggest that Smad1 phosphorylation on reoxygenation is dependent on the ligand binding. To examine the protective effect of BMP2 against injury during H/R, we performed an MTS assay. BMP2 promoted cardiomyocyte viability in a dose-dependent manner during the reperfusion phase but not during hypoxia alone (Figure 1C). In Figure 1D, Smad1 was phosphorylated 15 minutes after stimulation in a dose-dependent manner. These results indicate that BMP2 protects against the cardiomyocyte injury induced by H/R. Next, we performed an MTS assay of cardiomyocytes transfected with Ad-Smad1 or antisense Smad1 oligonucleotides (AS) under H/R. Ad-Smad1 promoted cell survival to a significant extent during the reperfusion phase and during hypoxia alone, as shown in Figure 1E. Phosphorylation of Smad1 was confirmed in cardiomyocytes 12 hours after infection with Ad-Smad1 (Figure 1F). Furthermore, to examine the importance of the Smad1 signaling pathway for BMP2-mediated survival effect in cardiomyocytes, we selectively inhibited this pathway by AS. Expression of Smad1 was significantly abolished in the presence of AS but not in the presence of control oligos (Figure 1G). The survival-promoting activity of BMP2 was strongly attenuated, consistent with the inhibition of Smad1 (Figure 1H). These findings suggest that the BMP2/Smad1 signaling pathway promotes survival of cardiomyocytes during H/R.

Next, we examined whether the survival-enhancing effect of BMP2/Smad1 was mediated by inhibition of apoptosis and performed DNA ladder and TUNEL analyses. As shown in Figure 2A, BMP2 and Ad-Smad1 significantly reduced the number of TUNEL-positive cells compared with controls. DNA ladder analysis also demonstrated that both suppressed H/R-induced nuclear fragmentation (Figure 2B). Because changes in mitochondrial function resulting from loss of mitochondrial membrane potential (Δψ) can reportedly activate the apoptotic pathway, we next ascertained whether H/R-mediated apoptosis is associated with loss of Δψ. Compared with untreated control cultures under normoxic conditions, exposure to H/R significantly decreased the red/green fluorescence intensity ratio (Figure 2C and 2D), suggesting that H/R results in loss of Δψ. Application of BMP2 or Ad-Smad1 before H/R significantly increased the red/green fluorescence intensity ratio of cardiomyocytes, indicating that BMP2/Smad1 attenuates mitochondrial membrane depolarization after H/R. We then observed the status of Bax, cytochrome c, and caspase3 in cardiomyocytes after administration of BMP2 or Ad-Smad1 before H/R and found that BMP2/Smad1 prevented mitochondrial cytochrome c release to cytosol, translocation of Bax translocation to mitochondria, and cleavage of caspase3 (Figure 2E).

As shown in Figure 2, BMP2 and overexpression of Smad1 each significantly inhibited H/R-deprived cardiomyocyte apoptosis. These results indicate that BMP2/Smad1 acts as a survival factor in neonatal rat cardiomyocytes at least in part by preventing apoptosis.

Figure 1. BMP2/Smad1 has cardioprotective effects against H/R in cardiomyocytes. A, B, Protein extracts from cultured neonatal cardiomyocytes were taken at various time points after treatment with BMP2 (80 ng/mL), during H/R (12/3 hours), and after pretreatment of BMP2, followed by placement in BMP2-free medium just before H/R [H/R BMP2(+) midst], and were immunoblotted with anti–phospho-Smad1 and –Cdk-4 antibodies. Results are representative of 3 independent experiments with nearly identical results. Cdk-4 blot is presented as a loading control. B, Quantitative analysis of Smad1 phosphorylation. Intensity of Smad1 phosphorylation was measured by densitometric scanning. Relative amounts of Smad1 phosphorylation are expressed as percentages of Cdk-4. n = 3. C, D, Cardiomyocytes were pretreated with BMP2 at various concentrations before H/R or hypoxia alone. C, Viability of cardiomyocytes was determined by MTS assay. Values are relative absorbances normalized to the mean absorbance of untreated control. n = 3. D, Phosphorylation of Smad1 in cardiomyocytes. After stimulation with various concentrations of BMP2 used in MTS assay for 15 minutes, cardiomyocytes were harvested and subjected to immunoblotting analysis with antibodies to phospho-Smad1 antibody. E, F, Cardiomyocytes were pretreated with an adenoviral vector expressing Smad1 (Ad-Smad1) before H/R or hypoxia alone. E, Viability of cardiomyocytes was determined by MTS assay. F, Cardiomyocytes were harvested 12 hours after infection with Ad-Smad1 and subjected to immunoblotting analysis with antibody to phospho-Smad1 antibody. Experiments were performed 3 times with similar results. G, H, Cardiomyocytes were pretreated with AS before H/R or hypoxia alone. After 16 hours of incubation in medium containing AS or control oligo, cardiomyocytes were treated with BMP2 (80 ng/mL) before H/R. G, Western blot analysis of Smad1 protein expression in cardiomyocytes treated with BMP2 after transfection of AS or control. One of 3 independent experiments is shown. H, Viability of cardiomyocytes was determined by MTS assay.
Antisense oligonucleotide of bcl-xL attenuates antiapoptotic effects of BMP2 and Ad-Smad1.

We previously reported that BMP2 and Smad1 induced bcl-xL expression and that antisense oligonucleotides against bcl-xL mRNA eliminated BMP2-mediated protection of cardiomyocytes during serum deprivation. To demonstrate the specificity of bcl-xL in the BMP2- or Smad1-dependent antiapoptotic effect in H/R injury, cardiomyocytes were treated with bcl-xL antisense oligonucleotides or bcl-xL sense oligonucleotides. Antisense oligonucleotides against bcl-xL mRNA significantly inhibited BMP2- or Smad1-mediated enhancement of cell survival compared with sense oligonucleotides (Figure 3A and 3B). These results suggest that bcl-xL induced by activation of BMP2/Smad1 signaling plays a key role in cardioprotection during H/R.

Characterization of Smad1TG

To examine whether the BMP2/Smad1 pathway plays a role in protection against I/R injury of the heart in vivo, we generated Smad1TG. We obtained 2 heterozygous founder lines of Smad1TG: line 7 (low copy number) and line 14 (high copy number). Figure 4A and 4B depicts myocardial smad1 mRNA and protein levels in lines 7 and 14 at 6 weeks of age. Levels of myocardial smad1 mRNA and protein corresponded to the number of copies of the transgene incorporated into the mouse genome. The level of phosphorylation of myocardial Smad1 was also higher in line 14 than in line 7.

To investigate whether overexpression of Smad1 affects signaling mediated by the other Smads, we checked the expressions of Smad3, Smad4, and Smad7 in Smad1TG. As shown in Figure 4C, expressions of Smad3 and Smad4 were...
comparable in Smad1TG and WT. Smad7 was not detected in either type of mouse by Western blotting (data not shown).

Furthermore, neonatal cardiomyocytes prepared from line 14 exhibited significantly increased phosphorylated Smad1 and survival activity during H/R compared with those from WT (Figure 4D and 4E). These findings suggest that myocardial Smad1 is spontaneously and functionally activated in Smad1TG heart. Because line 14 exhibited more activation of Smad1, we focused on further characterization of line 14.

To examine whether Smad1TG affects in vivo cardiac function, we performed blood pressure measurement and echocardiography in anesthetized mice. No differences were found in any parameters between Smad1TG and WT (see the Data Supplement). Thus, the global cardiac structure and function of Smad1TG were normal. We also performed histological examination, which revealed no cardiac abnormalities such as hypertrophy, fibrosis, or necrosis in Smad1TG (data not shown). Smad1TG at >6 weeks of age also exhibited no abnormalities. The lifespans of Smad1TG and WT were comparable.

Cardiac Smad1 Is Activated During I/R
Western blot analysis was performed to detect phosphorylation of cardiac Smad1 after I/R. In the ventricles of WT, Smad1 was transiently phosphorylated during 1-hour ischemia and again phosphorylated during 1-hour reperfusion. The level of Smad1 phosphorylation was less than that found in Smad1TG in basal conditions (Figure 5A and 5B). Interestingly, Bcl-xL was also induced in the heart during 1-hour reperfusion. Thus, I/R activated Smad1 and induced expression of Bcl-xL in the heart.

Apoptosis After I/R
We determined areas at risk (AAR) and infarcted area (IA) after I/R. No difference was found in AAR between Smad1TG and WT. However, a significant difference between them was found in the size of IA (pale white areas in Figure 6A, right). IA/AAR was significantly smaller in Smad1TG than in WT (Figure 6B). We examined apoptotic death of cardiomyocytes by TUNEL and DNA ladder analyses. I/R induced apoptosis and produced marked DNA ladder formation in WT. Smad1TG had significantly fewer
apoptotic cells and DNA fragmentations in the heart than did WT (Figure 6C and 6D). Cleavage of caspase3 was detected in WT but not in Smad1TG after I/R (Figure 6E). These findings suggest that Smad1 protects cardiomyocytes against I/R injury by reducing apoptotic cell death in the heart.

Bcl-xL Expression Is Increased in Smad1TG

To elucidate the mechanism of cardioprotective effect in Smad1TG, we examined whether expression of Bcl-xL was increased in Smad1TG. Northern and Western blot analyses revealed that levels of expression of bcl-xL mRNA and protein were markedly increased in Smad1TG but revealed no differences in levels of expression of bcl-2 mRNA, Bcl-2, Bad, Bax, or caspase8 proteins between Smad1TG and WT either after I/R or at rest (Figure 7A, 7B, and 7C). Moreover, the level of bcl-xL induction was increased to a greater extent in line 14 than in line 7 (Figure 7A), indicating that induction of bcl-xL was dependent on activation of Smad1. These results suggest that Smad1TG promotes cardiomyocyte survival through the induction of Bcl-xL.

β-Catenin Expression in Smad1TG After I/R

Recently, it has been reported that Smads and β-catenin are coactivators for the same transcriptional factors and that Wnt and BMP synergistically activated the expression Id2 and Msx1 genes.20,21 We therefore examined whether overexpression of Smad1 affected the Wnt/β-catenin signaling pathway in Smad1TG during I/R. Interestingly, β-catenin protein level was significantly increased in Smad1TG heart after I/R compared with WT (Figure 8A and 8B). On immunohistochemical analysis, β-catenin was found to have significantly accumulated in intercalated disks of Smad1TG after I/R compared with those in WT (Figure 8C and 8D). These findings suggest that Wnt signaling might play a role in reducing the size of infarction and the number of apoptotic cardiomyocytes in Smad1TG after I/R.

Discussion

The aim of our study was to characterize the protective role of BMP2/Smad1 signaling in adult heart. We demonstrated that Smad1 protected against I/R-induced injury of cardiomyocytes through the induction of Bcl-xL and β-catenin.
overexpression of Smad1 might play a role in the mechanism responsible for continuous activation of Smad1.

Our findings indicate that BMP2 and Smad1 suppressed H/R-induced mitochondrial defects, translocation of Bax to mitochondria, release of cytochrome c from mitochondria to cytosol, and apoptosis, suggesting that the Smad1 signaling pathway might be important for protection against apoptosis during H/R. With respect to the mechanisms of the antiapoptotic effect of BMP2/Smad1, we focused on Bcl-xL expression in cardiomyocytes, because we previously obtained direct evidence of promotion by BMP2 of cardiomyocyte survival through induction of Bcl-xL in conditions of serum starvation. Antisense oligonucleotides against bcl-xL mRNA reduced BMP2- or Smad1-mediated cytoprotection against H/R injury. Bcl-xL is known to inhibit the formation of active Bax and cytochrome c release from mitochondria in apoptotic cells. These observations indicate that induction of the bcl-xL gene in cardiomyocytes might be a key molecular event in BMP2- or Smad1-induced myocardial protection during H/R.

In Smad1TG, we observed for the first time that activation of Smad1 resulted in a decrease in infarct size and the number of apoptotic cardiomyocytes in mice subjected to I/R. We found that an increase in expression of Bcl-xL was more remarkable and that caspase3 was less activated in the heart of Smad1TG than in that of WT after I/R injury. The level of bcl-xL mRNA was increased to a greater extent in line 14 than in line 7, indicating that induction of bcl-xL is dependent on activation of Smad1. The findings of in vitro studies suggest that Smad1-induced Bcl-xL in Smad1TG might play an important role in protection against I/R injury in mice. However, the level of protection against I/R was comparable in lines 7 and 14 (data not shown). These findings suggest that the level of expression of bcl-xL is high enough in both lines of Smad1TG to protect cardiomyocytes against I/R. In previous studies, administration of hepatocyte growth factor or endothelin-1 could clearly reduce cardiac I/R injury. Induction of Bcl-xL by these substances has been suggested to be a mechanism of cardioprotection. These findings suggest that the increased expression of Bcl-xL observed in transgenic mice plays a role in decreasing apoptosis of cardiomyocytes.

A growing body of evidence points to the involvement of Wnt or frizzled (fz) in cardiac physiology. Members of the Wnt/fz signal transduction pathway are involved in cardiomyopathy and myocardial repair after myocardial infarction. The role of the Wnt/fz pathway in programmed cell death is a matter of debate. In physiological conditions, the level of β-catenin is regulated by Wnt signaling, which stabilizes cytosolic β-catenin. In the absence of Wnt signaling, β-catenin is phosphorylated by glycogen synthase kinase-3, ubiquinated, and degraded in proteasomes. Therefore, induction of BMP2 or its receptor by
the recent finding of accumulation of β-catenin in intercalated disks of cardiomyopathic hearts indicates that this molecule is responsible mainly for mediation of cell-cell adhesion rather than transcription of Wnt target genes in adult heart.25 Because the accumulation of β-catenin in intercalated disks could increase myocardial wall stiffness, alteration of β-catenin might play a role in decreasing infarct size in Smad1TG. However, future experiments will investigate how Smad1 could influence the regulation of Wnt/β-catenin signaling in heart during I/R.

In conclusion, we have demonstrated that genetic modification of myocardium with the smad1 gene confers protection against I/R injury. Induction of Smad1 expression could thus represent an attractive, novel, and effective therapeutic intervention for heart failure. However, whether the transient phosphorylation of Smad1 in vivo is essential for cardiac protection during I/R cannot be answered on the basis of our data alone. Cardiac-specific knockout of smad1 gene may be more informative.

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