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Exogenous High-Mobility Group Box 1 Protein Prevents Postinfarction Adverse Myocardial Remodeling Through TGF-β/Smad Signaling Pathway

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ABSTRACT

High-mobility group box 1 (HMGB1) has been reported to attenuate ventricular remodeling, but its mechanism remains mostly unresolved. Transforming growth factor-beta (TGF-β) is a crucial mediator in the pathogenesis of post-infarction remodeling. Our study focused on the effects of HMGB1 on ventricular remodeling, and explored whether or not these effects were depended upon the TGF-β signaling pathway. Rats underwent coronary artery ligation. An intramyocardium injection of phosphate buffered saline (PBS) with or without HMGB1 was administered 3 weeks after myocardial infarction (MI). At 4 weeks after the treatment, HMGB1 significantly increased the left ventricular ejection fraction (LVEF) ($P < 0.05$), decreased the left ventricular end diastolic dimension (LVEDD; $P < 0.05$), left ventricular end systolic dimension (LVESD) ($P < 0.05$) and the infarct size ($P < 0.05$) compared with control group. The expressions of collagen I, collagen III, and tissue inhibitor of metalloproteinase 2 (TIMP2) were also decreased, while the matrix metalloproteinases 2 (MMP2) and MMP9 expressions were upregulated by HMGB1 injection ($P < 0.05$) compared with control group. No effect on TIMP3 was observed. Furthermore, TGF-β1 and phosphor-Smad2 (p-Smad2) were significantly suppressed and Smad7 was increased in HMGB1-treated group ($P < 0.05$) compared with control group, no effects on p-Smad3 and p-p38 were observed. HMGB1 also upregulated Smad 7 expression and decreased the level of collagen I on cardiac fibroblasts ($P < 0.05$). Silencing of Smad7 gene by small interfering RNA abolished the fibrogenic effects of HMGB1 on cardiac fibroblasts ($P < 0.05$). These finding suggested that HMGB1 injection modulated ventricular remodeling may function through the possible inhibition of TGF-β/Smad signaling pathway. J. Cell. Biochem. 114: 1634–1641, 2013.

KEY WORDS: HMGB1; TGF-β; SMAD; MYOCARDIAL INFARCTION

Cardiac remodeling after MI results in ventricular dysfunction and heart failure, which contributes to a poor outcome and high mortality. Previous reports have indicated that the inflammatory and the excessive deposition of extracellular matrix (ECM) including basic structural proteins such as collagen, laminin, and fibronectin play an important role in the pathogenesis of ventricular remodeling [Aukrust et al., 2005; Matsui et al., 2010]. Modulation of inflammation and ECM deposition may be an effective strategy to inhibit the process of ventricular remodeling fowling MI.

High-mobility group box 1 (HMGB1) is a nuclear protein which either actively secreted by activated immune cells or passively released from necrotic cells [Park et al., 2011]. HMGB1 as a proinflammatory cytokine, plays an important role in the modulation of inflammation through its interaction with toll-like receptor (TLR) 4 and 9 and the receptor for advanced glycation end products (RAGE) [Lotze and Tracey, 2005]. HMGB1 is reported to both amplify [Erlandsson and Andersson, 2004] and attenuate the inflammation [Popovic et al., 2006]. The previous studies demonstrated that HMGB1 can modulate cardiac remodeling via reducing the local inflammatory response after MI [Takahashi et al., 2008]. While in another study, HMGB1 activated proinflammatory pathways and enhanced ischemia/reperfusion injury. HMGB1 is also reported to have the potential to induce myocardial regeneration [Limana et al., 2005] and attenuate fibrosis in the post-MI failing myocardium [Takahashi et al., 2008]. However, the mechanism by which HMGB1 attenuates fibrosis and prevents post-infarct ventricular remodeling has not yet been studied in detail.

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Transforming growth factor-beta (TGF-β) belongs to a superfamily of proteins that serve central roles in infracted myocardium repair and remodeling. Expressions of TGF-β are significantly upregulated in the scar and border zone area after MI. TGF-β may play an important role in regulating the post-infarction inflammatory response by suppressing expression of pro-inflammatory cytokines and chemokines in the infarcted myocardium [Werner et al., 2000]. Furthermore, TGF-β is correlated with fibrous tissue deposition by inducing ECM protein synthesis following MI [Saltis et al., 1996]. Increased TGF-β levels in the remodeling heart are associated with activation of TGF-β signaling [Xia et al., 2009]. However, the relationship between HMGB1 and TGF-β/Smad signaling pathway remains unknown. This encouraged us to investigate whether HMGB1 attenuate cardiac remodeling through the inhibition of TGF-β/Smad signaling pathway.

MATERIALS AND METHODS

MODEL OF MYOCARDIAL INFARCTION AND HMGB1 APPLICATION

Male Sprague Dawley (SD) rats (200–250 g) were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg). The left anterior descending coronary artery was ligated between the left atrial appendage and the right ventricular outflow with a 6-0 silk suture through a left thoracotomy. Local wall motion abnormality and pallor of the infracted left ventricle (LV) were considered as successful MI models. Rats in the shame-operated group underwent thoracotomy without LAD ligation. Forty-five MI animals were randomly assigned to tow groups: MI control group, HMGB1 implantation group. Fifteen rats underwent sham operation. Three weeks after inducing myocardial infarction (MI), 100 μl phosphate buffered saline (PBS) without HMGB1 or containing 2.5 μg HMGB1 [Takahashi et al., 2008] was injected into four sites (25 μl per site) of the ventricular wall bordering the viable myocardium through a 32-gauge needle.

ASSESSMENT OF LV FUNCTION

The LV function was assessed by echocardiography using a sequoia 512 and 15 MHz probe at 4 weeks after injection. The following parameters were derived from the M-mode tracing: left ventricular end systolic dimension (LVESD) and left ventricular end diastolic dimension (LVEDD) were measured from at least three consecutive cardiac cycles. left ventricular ejection fraction (LVEF) was calculated using the following formula: $LVEF = [(LVEDD^3 - LVESD^3)/LVEDD^3] \times 100\%$, and the results were expressed as percentage.

HISTOLOGICAL ASSESSMENT

Four weeks after treatment, a lethal dose of sodium pentobarbital was injected to kill the rats, and the hearts were excised. Ventricles were fixed in 10% (v/v) buffered formalin solution and embedded in paraffin, sectioned at 4 μm and stained with Masson-trichrome. Infarct size was calculated from the percentage of infracted area to total LV area using Image analysis software.

REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was isolated from myocardial tissue into Trizol reagent (Boehringer Mannheim, Mannheim, Germany). To determine the levels of expression of TGF-β1 and β-actin, quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed with Light Cycler (Roche, Mannheim, Germany) by using 1μg total RNA as described previously [Andrassy et al., 2006]. The primer sequences were as follows: TGF-β1 forward, 5′-CTTCAGCTCCACAGGAAGA ACTG-3′, TGF-β1 reverse, 5′-CACGATCATGTTGGACAACTGCTCC-3′; β-actin forward, 5′-CCCTAAGGCCAACCGTGA AA-3′, and β-actin reverse, 5′-ACGACCAAGGCATACAGGGA-3′.

Standard curves were established with SYBR Green I kit (Roche, Basel, Switzerland). All data were quantified by the use of the comparative cycle threshold method, normalized to β-actin.

RT-PCR

RNA was extracted with TRIzol (Invitrogen). Preamplification system was used to reverse-transcribe total RNA (1 μg) into complementary DNA according to manufacturer's instructions (Invitrogen). An aliquot (2 μl) of the reverse transcription reaction was subjected to 39 polymerase chain reaction cycles: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, in the presence of 50 pmol of each primer, 1.5 mmol/L $MgCl_2$, 200 mmol/L dATP, dCTP, dGTP, and dTTP each, and 2.5 U of Amplitaq polymerase (Invitrogen). Sequences of the primers were: collagen III 5′-CTGGTCCTGTTG GTCCATCT-3′ and 5′- ATGCCATTAGAGCCACGTTC-3′; actin 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCT GTA-3′. The polymerase chain reaction products were electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

WESTERN BLOT ANALYSIS

Total protein was extracted from mouse heart tissue with ice-cold lysis buffer as described previously [Limana et al., 2005]. After measuring protein concentration, 50 μg protein were subjected to SDS–PAGE electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was probed with primary antibody 4°C overnight. The antibodies and the dilutions were as follows: collagen I (1:300), collagen III (1:300), matrix metalloproteinases 2 (MMP2; 1:300), MMP9 (1:300), tissue inhibitor of metalloproteinase 2 (TIMP2; 1:300), TIMP3 (1:300; Santa Cruz); Smad2 (1:500), p-Smad2 (1:500), and TGF-β1 (1:500), Smad3(1:500), p-Smad3 (1:500), Smad7 (1:500), p-p38 (1:500), p38 (1:500; Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-coupled rabbit or mouse IgG (1:2,000, Beijing Zhongshan Biotechnology, China) was used as secondary antibodies. Blots were visualized with the use of an enhanced chemiluminescence kit (Amersham Biosciences, Inc., Piscataway, NJ). Developed films were scanned and Image J software was used to perform quantitative analysis. GAPDH was used as control.

CARDIAC FIBROBLAST CULTURE

Cardiac fibroblasts cultures were established from ventricular tissue of male SD rats weighing 250–300 g. The ventricles were isolated, minced, pooled, and placed in a solution of 100 U/ml collagenase I and 0.1% trypsin. Cells dissociated in the first treatment were

discarded. Digested fibroblasts from three more digestion periods were pooled, pelleted, and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin, and 10% fetal bovine serum. After a 60-min period of attachment to uncoated culture plates, cells that were weakly attached or unattached were rinsed free and discarded. The attached cells were cultured in an incubator with 5% CO_2 at 37°C. The culture medium was changed every other day. These cultures contained 95% cardiac fibroblasts as indicated by positive for procollagen type 1, which is a major protein product of fibroblasts, and negative for smooth muscle actin and von Willebrand factor. When the cardiac fibroblast culture was about 80% confluence, they were serum-starved for 24 h. They were then used in the following studies.

(1) Cardiac fibroblasts was treated with: (A) 0.005% dimethylsulfoxide (DMSO) (control group); (B) 10 ng/ml TGF-β1 for 6h; (C) 100 ng/ml HMGB1 + 10 ng/ml TGF-β1 for 6 h. Cells were treated with HMGB1 at 30 min before TGF-β1 stimulation. Cells were then harvested. The protein expression of Smad7 and collagen I was determined by Western blotting.

(2) The Silencer small interfering (si)RNAs targeting Smad7 were synthesized according to rat-specific sequences. As a negative control, a non-targeting scrambled siRNA (control siRNA) was used. Cardiac fibroblasts were transfected with 400 pmol of siRNA using Lipofectamine 2000 in a 6-well plate according to the instructions of the manufacturer. Clones of Smad7 siRNA that presented at least 90% inhibition of target genes were chosen for further analysis. After 24 h of transfection, cells were treated with or without HMGB1 (100 ng/ml) for 6 h. Levels of Smad7 and collagen I expression were determined by Western blot.

STATISTICAL ANALYSIS

Values are expressed as mean ± SE. One-way analysis of variance (ANOVA) and the Newman–Keuls–Student *t*-test followed by Bonferroni adjustment for multiple comparisons were used for comparisons of >2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

MORTALITY

3.3% (2/60) rats died of anesthesia before the surgery, 11.7% (7/60) rats died due to acute heart failure within 48 h after LCA ligation. Three weeks after LCA ligation, the surviving 36 MI rats were randomly assigned to receive either HMGB1 or PBS injection into the peri-infarct zone. After either injection, there was 100% survival in all groups.

CARDIAC FUNCTION AND LEFT VENTRICULAR REMODELING AFTER HMGB1 INJECTION

Ejection fraction was decreased and LV volume was increased in all acute MI animals, LVEF was higher ($P < 0.05$; Fig. 1A), LVESD and LVEDD were smaller ($P < 0.05$; Fig. 1B,C) in the HMGB1-treated group compared to the control-treated group at 4 weeks after MI. The infarct size percent was significantly reduced ($P < 0.05$; Fig. 2B,C,G) at 4 weeks after HMGB1 injection compared with the control group.

HMGB1 reduces collagen deposition and enhances collagenolytic activity in the peri-infarct region. The expressions of collagen I, TIMP2, and collagen III in the peri-infarct area were evidently decreased ($P < 0.05$; Figs. 3A,B, and 4A–D) of the HMGB1-treated group compared with control-operated group at 4 weeks after injection. There was no significant difference in TIMP3 expression between control and HMGB1 group. MMP2 and MMP9

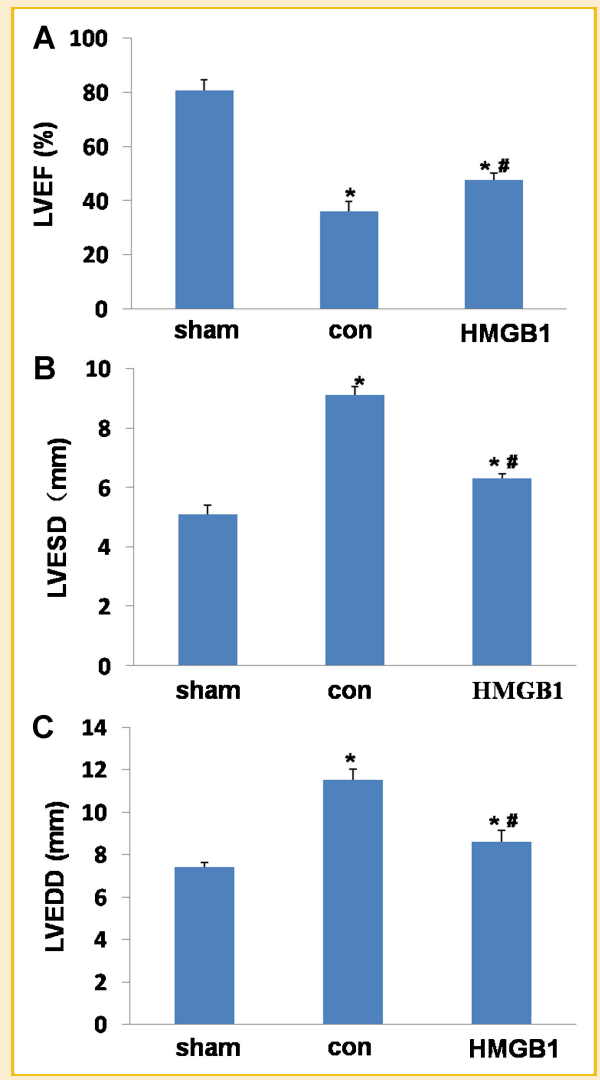

Fig. 1. Changes in cardiac parameters by HMGB1 injection. Cardiac parameters including left ventricular ejection fraction (LVEF), left ventricular end systolic dimension (LVESD) and left ventricular end diastolic dimension (LVEDD) were assessed by echocardiography. At 4 weeks after injection, LVEF was higher A, LVESD and LVEDD were smaller in the HMGB1 group than that in the control group (B and C). Data are expressed as means ± SEM. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus con, n = 15 (sham) and n = 15 (con, HMGB1).

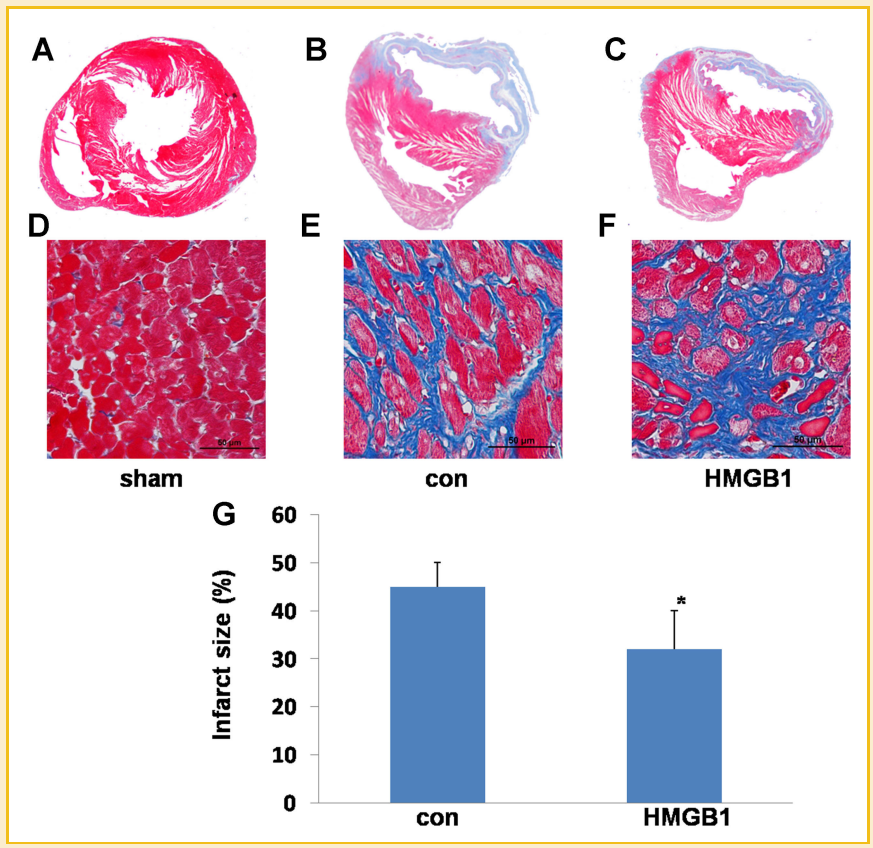

Fig. 2. Evaluation of the infarct size after myocardial infarction. The infarction area is stained blue. Sham group (A: 10×, D: 400×) compared with controls, injection of HMGB1 reduced infarct size (B: 10×, C: 10×, G), and showed lower collagen content in the peri-infarct area (E: 400×, F: 400×) 4 weeks after injection. Data are expressed as means ± SEM. $^{*}P < 0.05$ versus con, n = 8 in each group.

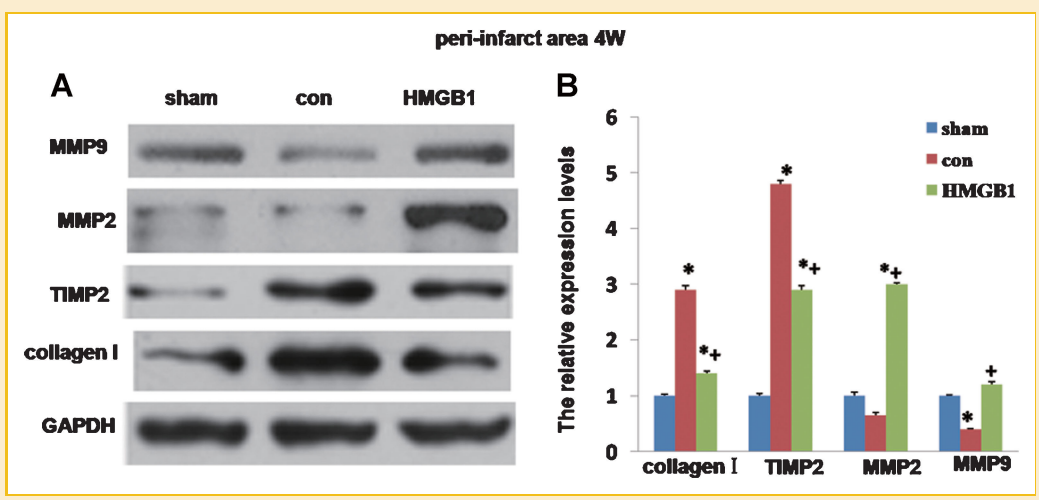

Fig. 3. Western blot analyses of collagen I, TIMP2, MMP2, and MMP9 expressions (A and B). The expressions of TIMP2 and collagen I were decreased, while the expressions of MMP2 and MMP9 were increased by HMGB1 in the peri-infarct area at 4 weeks. Data are expressed as mean + SEM. $^{*}P < 0.05$ versus sham, $^{+}P < 0.05$ versus con, n = 8 in each group.

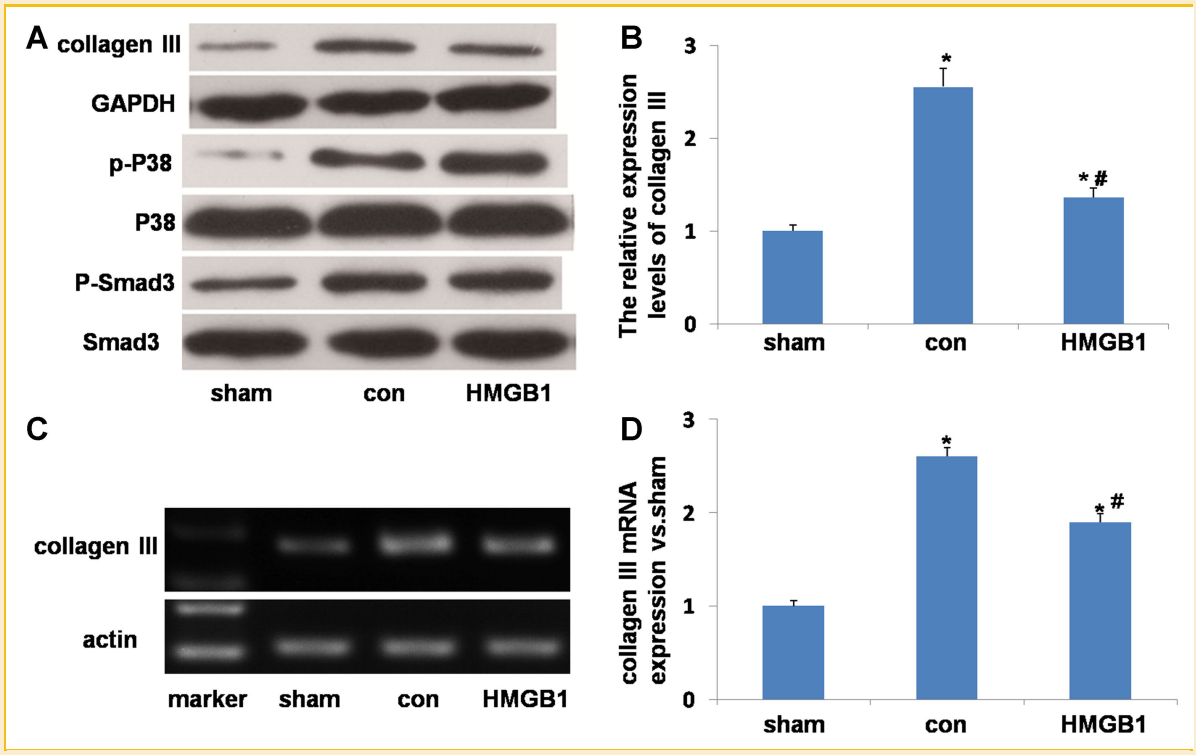

Fig. 4. Both the protein and the mRNA expression of collagen III were decreased by HMGB1 in the peri-infarct area of MI rats at 4 weeks compared to the control group (A–D). No effect on the levels of p-Smad3 and p-p38 were observed. Data are expressed as mean + SEM. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus con, n = 5 in each group.

expressions were increased in HMGB1 group compared to control group ($P < 0.05$; Fig. 3A,B).

Effects of HMGB1 on TGF-β signaling pathway in vivo. Real-time PCR demonstrated that the mRNA expression of TGF-β1 in the peri-infarct area was upregulated in MI rats compared with sham group at 4 weeks after injection ($P < 0.05$; Fig. 5). Compared with control-treated rats, the TGF-β1 mRNA expression was down-regulated in HMGB1-treated rats ($P < 0.05$; Fig. 5). Western blot analyses suggested that the expression levels of TGF-β1, and p-Smad2 protein in the peri-infarct area were significantly increased in the control group compared with the sham group at 4 weeks after injection ($P < 0.05$; Fig. 6A–D). This increase was evidently attenuated by HMGB1 injection ($P < 0.05$; Fig. 6A–D). Smad7 expression was increased in HMGB1 group compared to control and sham group ($P < 0.05$; Fig. 6C,D). There was no significant difference in p-Smad3 and p-p38 expression between control and HMGB1 group (Fig. 4A).

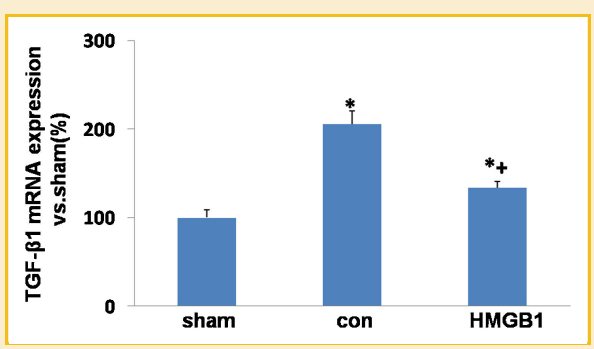

Fig. 5. Real-time quantitative reverse transcriptase-polymerase chain reaction analyses of TGF-β1 expression. The mRNA expression of TGF-β1 was upregulated in the peri-infarct area of MI rats at 4 weeks compared to the sham rats, this increase was evidently attenuated after HMGB1 injection. Data are expressed as mean + SEM. $^{*}P < 0.05$ versus sham, $^{+}P < 0.05$ versus con, n = 8 in each group.

Role of HMGB1 on TGF-β signaling pathway in vitro. Treatment of cardiac fibroblasts with TGF-β1 significantly decreased the protein expression of Smad7 and increased collagen I protein expression. These effects were attenuated by HMGB1 ($P < 0.05$; Fig. 7A,B).

EFFECTS OF SMAD7 GENE SILENCING BY SIRNA ON HMGB1-ATTENUATED COLLAGEN SYNTHESIS IN CARDIAC FIBROBLASTS

Transfection with siRNAs targeting Smad7 significantly down-regulated the expression level of Smad7 (>90%), as measured by Western blotting (Fig. 8A). HMGB1 caused a significant decrease in collagen I expression ($P < 0.005$) and a increase in Smad7 expression ($P < 0.005$; Fig. 8A,B) in cardiac fibroblasts transfected with control siRNA. The decrease in collagen I expression in the fibroblasts by HMGB1 was significantly attenuated by knocking-down Smad7 ($P < 0.05$; Fig. 8A,B).

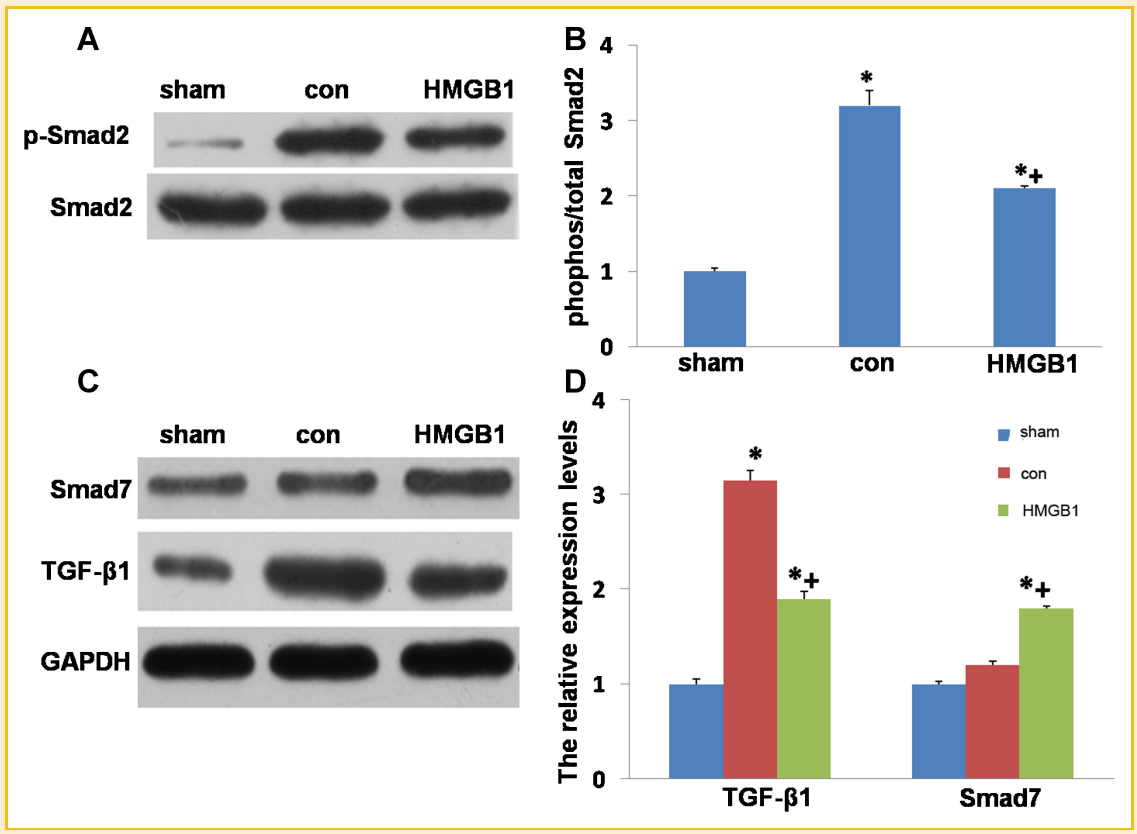

Fig. 6. The effects of chronic treatment with HMGB1 on the expressions of TGF-β1, p-Smad2, and Smad7 were analyzed by Western blots. TGF-β1(C and D), and p-Smad2/Smad2 (A and B) expressions in the peri-infarct area were significantly decreased and Smad7 (C and D) expression was increased by HMGB1 compared with the control group at 4 weeks after myocardial infarction. Data are expressed as mean + SEM. $^{*}P < 0.05$, versus sham group, $^{+}P < 0.05$ versus con, n = 8 in each group.

DISCUSSION

In this study, we examined the role of HMGB1 on TGF-β/Smad signaling pathway after MI. The major finding of this study include the following: (1) we demonstrated that HMGB1 significantly improved global cardiac function and attenuated LV remodeling; (2) the levels of collagen I, collagen III, and TIMP2 were reduced while the MMP2 and MMP9 expressions were upregulated by HMGB1 following MI; (3) HMGB1 caused a significant down-regulation of TGF-β1 levels and inhibition of p-Smad2 expression, and increased

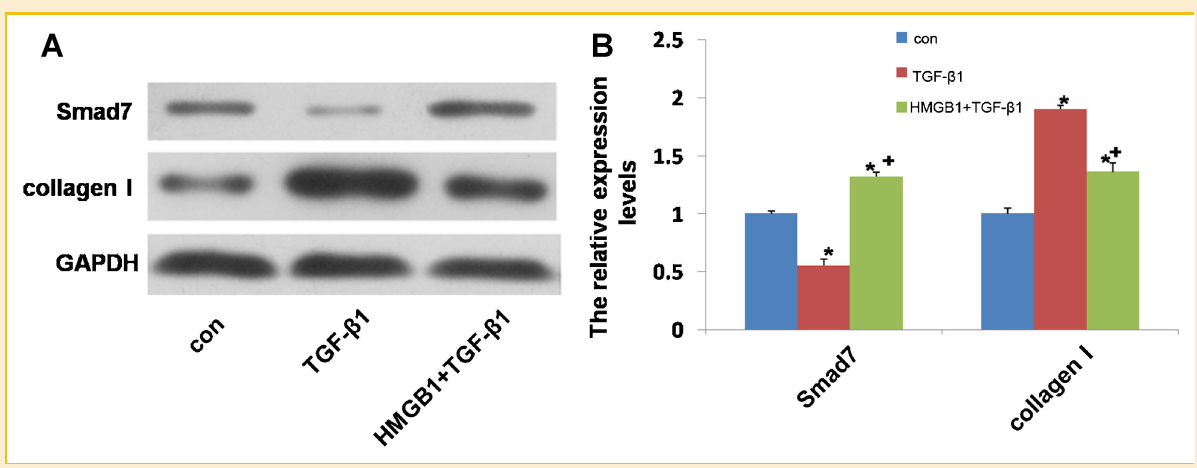

Fig. 7. HMGB1 induces Smad7 expression and blocks TGF-β1 induced collagen I expression by cardiac fibroblasts in vitro. Western blot analysis for Smad7 and collagen I protein expression. (A and B). Data represent mean + SEM for at least three independent experiments. $^{*}P < 0.05$ versus control, $^{+}P < 0.05$ versus TGF-β1 treated cells.

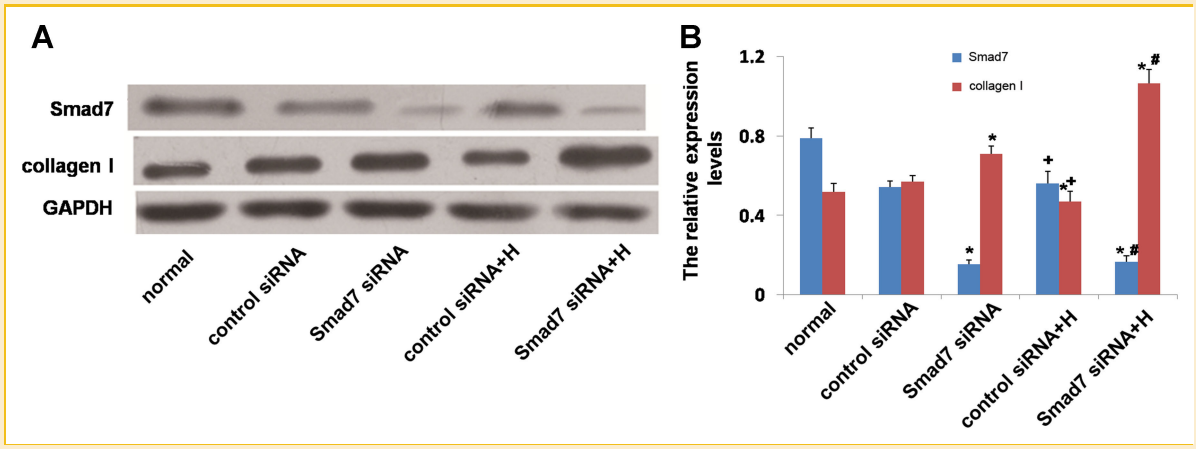

Fig. 8. Effect of Smad7 knockdown on collagen I expression in cardiac fibroblasts. Western blot analysis for collagen I and Smad7 expression in cardiac fibroblasts without transfection (normal) or transfected with control siRNA or Smad7 siRNA when exposed to HMGB1 (control siRNA + H or Smad7 siRNA + H) or without HMGB1 (control siRNA or Smad7 siRNA; A and B). Smad7 siRNA successfully knocked down the expression of Smad7 (A). HMGB1 decreased collagen I expression and increased Smad7 expression (A and B) in cardiac fibroblasts transfected with control siRNA. The decrease in collagen I expression in the fibroblasts by HMGB1 was significantly attenuated by knocking-down Smad7 (A and B). Data represent mean + SEM for at least three independent experiments. $^{*}P < 0.05$ versus control siRNA group, $^{+}P < 0.05$ versus Smad7 siRNA group, $^{\#}P < 0.05$ versus control siRNA + H group.

Smad7 activity, but p-Smad3 and p-p38 expressions were not changed; (4) in isolated adult rat cardiac fibroblasts, HMGB1 also caused an upregulation of Smad7 expression and decreased the level of collagen I; (5) silencing of Smad7 gene by siRNA abolished the downregulation of collagen I synthesis by HMGB1 on cardiac fibroblasts. These results strongly suggest that downregulation of an activated TGF-β/Smad2 signaling pathway and upregulation of Smad7 may be a novel mechanism for HMGB1 to attenuate left ventricular remodeling after MI.

MI caused by the occlusion of a coronary artery leads to cardiac myocyte death, tissue loss, and scar formation. Therefore, effective strategies to induce cardiac regeneration and inhibit fibrosis tissue synthesis represent promising treatment for ventricular remodeling. HMGB1 is a nuclear DNA-binding protein secreted into the extracellular milieu from necrotic and inflammatory cells, and acts as a cytokine with multiple functions. Local administration of HMGB1 facilitated myocardial cell regeneration [Limana et al., 2005], enhanced angiogenesis [Kitahara et al., 2008], and attenuated inflammation [Takahashi et al., 2008] following MI which consequently improved myocardial function. Moreover, HMGB1 enhanced MMP2 and MMP9 activity and decreased TIMP-3 levels which may contribute to its attenuation of ECM deposition and ventricular remodeling [Limana et al., 2011]. In our research, we found HMGB1 can significantly reduce the expression of collagen I, collagen III and decreased the expressions of TIMP2 while increased MMP2 and MMP9 expressions.

TGF-βs are pleiotropic cytokines which have variety cell functions, such as regulating inflammation, ECM deposition, cell proliferation, differentiation, and growth [Lawrence, 2001]. TGF-β expression is upregulated in experimental models of MI and plays an important role in the pathogenesis of cardiac remodeling and fibrosis. The previously study reported that the TGF-β has the potential to up-regulate the transcription of several genes that are important for ECM formation, such as the collagen I and the collagen III [Verrecchia and Mauviel, 2002]. Furthermore, TGF-β stimulation induces myofibroblast differentiation and enhances ECM protein synthesis by inhibiting MMP expression and inducing synthesis of protease inhibitors, such as tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitor (PAI)-1 [1] [Eghbali et al., 1991; Schiller et al., 2004; Verrecchia et al., 2001]. To explain above findings, we further investigate whether HMGB1's beneficial effects are associated the regulation of TGF-β expression. In our study, TGF-β1 was upregulated after MI. HMGB1 administration appears to have the potential to down-regulate TGF-β1 expression.

TGF-β1 has been noted to exert biological effects through a Smad-dependent or a Smad-independent signaling pathway [Rahimi and Leof, 2007]. Previously study indicated that blockade of Smad2 or Smad3 activation and overexpression of exogenous I-Smad7 may result in inhibition of TGF-β induced collagen deposition which may contribute to cardiac fibrosis in the remodeling myocardium [Wang et al., 2007]. In the present study, we further tested the hypothesis that HMGB1 inhibits TGF-β1 stimulated ECM expression through Smad signaling parthway. We observed that cardiac expression of p-Smad2 in the peri-infarct area was strongly upregulated in control mice 4 weeks post-MI. This upregulation was significantly inhibited by HMGB1 injection. While the Smad7 expression was increased after the implantation of HMGB1 compared with control group. Furthermore, HMGB1 also upregulated Smad7 expression and decreased collagen I expression in vitro, but HMGB1 on pro-fibrogenic effects are omitted during smad7 knock-down. No effect on p-Smad3 expression was observed. Besides Smad-mediated transcription, we also detected the p-p38 expression, but no significant change of p-p38 was observed after HMGB1 implantation compared with control group. These findings suggested that HMGB1 reduced cardiac remodeling and decreased collagen synthesis and that these effects may possibly associate with the TGF-β/Smad signaling pathway.

In summary, the present study provides both in vivo and in vitro experimental evidence that HMGB1 attenuate collagen I and collagen III deposition. These effects are possibly mediated through the downregulation of the TGF-β/Smad2 signaling pathway and upregulation of Smad7 expression. These findings may lead to a novel potential strategy to prevent ventricular remodeling and enhance heart function after coronary artery occlusion. Further detailed studies are necessary to understand the mechanism of how HMGB1 affect TGF-β/Smad signaling pathway and ultimately attenuate cardiac remodeling, and to define the therapeutic potential of HMGB1 in the cardiac healing and remodeling processes after MI.

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