European Review for Medical and Pharmacological Sciences 2020; 24: 1350-1356

Effect of TGF-β1 on myocardial cell apoptosis in rats with acute myocardial infarction *via* MAPK signaling pathway

X.-G. ZHANG[1], Y. WEI[2], J. JIANG[3], L. WANG[4], H.-Y. LIANG[5], C.-B. LEI[6]

[1]Department of Clinical Laboratory, Affiliated Hospital of Weifang Medical College, Weifang, China
[2]ECG Room, Dezhou People's Hospital, Dezhou, China
[3]Department of Nursing, the Third People's Hospital of Qingdao, Qingdao, China
[4]Department of Clinical Laboratory, Qingdao Hospital of Shandong Province, Qingdao, China
[5]Department of Pharmacy, the People's Hospital of Zhangqiu Area, Jinan, China
[6]Department of Clinical Laboratory, Zibo Central Hospital, Zibo Central Hospital Affiliated to Binzhou Medical University, Zibo, China

Xuguang Zhang and *Ying Wei* contributed equally to the work

Abstract. – OBJECTIVE: Transforming growth factor beta 1 (TGF-β1) can promote myocyte hypertrophy, thus playing an important role in ventricular remodeling after myocardial infarction (MI).

MATERIALS AND METHODS: In this study, the model of MI was established in rats through ligating the left anterior descending coronary artery. Subsequently, the messenger ribonucleic acid (mRNA) and protein expression levels of TGF-β1 in myocardial cells in both model group and sham operation group were determined. The effects of TGF-β1 treatment on myocardial cell apoptosis in MI rats were explored. Moreover, the changes of mitogen-activated protein kinase (MAPK) signaling pathway in rats with acute MI were verified. In addition, the protein expressions of phosphorylated-MAPK kinases 3/6 (p-MKK3/6) and MKK3/6 in myocardial cells of the two groups were analyzed.

RESULTS: The mRNA and protein expression levels of TGF-β1 in myocardial cells of acute MI rats were significantly higher than those in the sham operation group ($p<0.01$). After treatment with TGF-β1, the expression level of B-cell lymphoma 2 (Bcl-2) associated X protein (Bax) was obviously down-regulated. The Bax/Bcl-2 ratio was notably lower than that in control group ($p<0.01$). Meanwhile, the proportion of apoptotic cells decreased remarkably ($p<0.01$). In the model group, no evident change was observed in the protein expression level of MKK3/6, whereas the levels of p-MKK3/6 were prominently up-regulated ($p<0.01$).

CONCLUSIONS: TGF-β1 can activate MKK3/6 in the MAPK signaling pathway to resist the apoptosis of myocardial cells in acute MI rats.

Key Words:
TGF-β1, MAPK signaling pathway, Acute myocardial infarction (AMI), Myocardial cell apoptosis.

Introduction

Acute myocardial infarction (MI) has posed enormous threats to human health, currently. In America, there are about 550,000 new cases of acute MI annually, with 200,000 cases of recurrent acute MI[1]. Ischemic heart disease has become a leading cause of disease burden[2]. With the development of economy, the incidence rates of cardiovascular diseases and acute MI are increasing in developing countries[3]. In recent years, studies[4] have suggested that the incidence rate of acute MI is inversely proportional to incomes.

Transforming the growth factor-beta 1 (TGF-β1) is an important cell growth factor. It exerts many biological effects, such as growth inhibition, bone remodeling, and incorporation into extracellular matrix[5]. In the lung, the increase in the bronchoalveolar lavage fluid is correlated with the development of edema in a time-dependent manner in the model of Bleomycin-induced acute lung injury[6]. Additionally, in the adenovirus-mediated gene delivery, the overexpression of active TGF-β1 in lung tissues of rats induces the initial-phase edema around the vessels and bronchi[7]. With the prolongation of time, the long-term and severe pulmonary fibrosis developed due to the persistent expression of this active factor[7].

Corresponding Author: Chengbin Lei, MM; e-mail: fipjwve@163.com

Pittet et al[8] have provided the latest evidence for the importance of TGF-β1 in acute lung injury. Meanwhile, some researchers have found that the expression of chimeric TGF-β1 receptors that prevent TGF-β1 from interacting with its receptor can avoid the Bleomycin-induced pulmonary edema[6]. All these findings reveal that TGF-β1 has an effect on the increase of vascular permeability under such pathological conditions leading to pulmonary edema and/or vascular injury.

After MI, TGF-β1 is significantly up-regulated[9,10]. It can also form a signal transduction network with the renin-angiotensin system, thereby promoting cardiac remodeling[11]. TGF-β1 induces the expression of fetal genes in myocardial cells and activates the cardiac fibroblasts to produce extracellular matrix proteins[11]. With a heteromeric complex of two serine-threonine kinase receptors, TGF-β1 initiates the biological responses[10]. Furthermore, it transduces signals to MKK3/6 and p38 MAPK, which are members of the mitogen-activated protein kinase (MAPK) kinase family[11,12].

In the present study, we explored whether TGF-β1 affected myocardial cell apoptosis in MI rats via the MAPK signaling pathway based on the acute MI rat model.

Materials and Methods

Materials

Xylazine hydrochloride was purchased from Shenzhen ChemStrong Scientific Co., Ltd. (Shenzhou, China), 2, 3, 5-triphenyltetrazolium chloride from Solarbio (Beijing, China), RNeasy mini kit from Qiagen (Hilden, Germany), SuperScript III First-strand synthesis kit from Invitrogen (Carlsbad, CA, USA), and the anti-TGF-β1 antibody, anti-B-cell lymphoma 2 (Bcl-2) antibody, anti-Bcl-2 associated X protein (Bax) antibody, anti-MKK3/6 antibodies, and anti-phosphorylated-MKK3/6 (p-MKK3/6) antibodies from Abcam (Cambridge, MA, USA).

Establishment of Acute MI Model in Rats

The chest of rats was cut opened via anterior thoracotomy to expose the heart. Subsequently, the proximal left anterior descending coronary artery was ligated using suture threads at about 2 mm away from the distal aorta. The regional purpura on the surface of the myocardium was observed to verify the success of ligation. Then, the heart was replaced, and the incision was closed. In the sham operation group, the chest of the rats was opened using the same method, without ligation of the coronary artery. MI area and the ratio of heart weight/body weight were determined to judge whether the model was successfully established. This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Weifang Medical College Animal Center.

Determination of Heart Weight/body Weight Ratio and MI Area

After anesthesia with xylazine hydrochloride and execution through the injection of potassium chloride, the heart of rats was taken out. Subsequently, the heart and body were weighed, and the ratio was calculated. Next, the heart was sectioned, embedded, and frozen for use. Finally, the infarction area was determined via 2, 3, 5-triphenyltetrazolium chloride staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To analyze the messenger ribonucleic acid (mRNA) level of TGF-β1, the total RNA was isolated from myocardial tissues using the RNesy mini kit. The extracted RNA was then reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the SuperScript III First-strand synthesis kit. The mRNA expression level of genes was measured via RT-PCR. After that, the standard curve was plotted using the amplification results of cDNA recombinant plasmids with known copy numbers. The cyclic threshold (Ct) was determined, and the relative level of mRNAs was calculated based on Ct value. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The primer sequences used in this study were as follows: TGF-β1 primer: sense 5′-TGAACCGGCCTTTCCTGCTTCTCATG-3′ and anti-sense 5′-TGAACCGGCCTTTCCTGCTTCTCATG-3′ and GAPDH primer: sense 5’-GAGTCCACTGGCGTCTTCA-3′ and anti-sense 5’-GGGGTGCTAAGCAGTTGG-3′.

Western Blotting

The radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in heart tissues. The concentration of the extracted protein was determined by the bicinchoninic acid (BCA) method. The protein samples were electrophoresed on polyacrylamide gels and transferred onto polyvi-

nylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with anti-TGF-β1 antibody, anti-Bcl-2 antibody, anti-Bax antibody, anti-MKK3/6 antibodies, and anti-p-MKK3/6 antibodies overnight. On the next day, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies. The immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system.

Flow Cytometry

Cell apoptosis was determined using the flow cytometry. The myocardial cells were isolated and cultured until logarithmic phase. Then, the cells were made into single-cell suspension and inoculated into 6-well plates. After treatment with TGF-β1, the cells were washed with Phosphate-Buffered Saline (PBS), followed by re-suspension in 500 μL of annexin V binding buffer. Subsequently, the cell suspension was added with 5 μL of annexin V-fluorescein isothiocyanate (FITC) solution and 5 μL of Propidium Iodide (PI) solution. The mixture was incubated in a dark place at room temperature for 10 min. Finally, early cell apoptosis was evaluated by flow cytometry.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The experimental data were expressed as $\bar{\chi}\pm$SD (standard deviation). The *t*-test was adopted to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Establishment of Acute MI Rat Model

The model of MI was successfully established in rats *via* ligating the left anterior descending coronary artery. The success of infarction modeling was evaluated by the assessment of heart weight/body weight ratio and infarction area. The results showed that, compared with the sham operation group, the model group exhibited significantly elevated heart weight/body weight ratio ($p<0.05$) (Figure 1A). Meanwhile, MI area was remarkably higher in the model group than in the sham operation group ($p<0.01$) (Figure 1B).

Expression of TGF-β1 in Myocardial Cells in the Acute MI Rat Model

The expression of TGF-β1 in myocardial cells in both model group and sham operation group was detected. QRT-PCR and Western blotting results demonstrated that the mRNA and protein expression levels of TGF-β1 in myocardial cells of the model group were remarkably higher than in the sham operation group ($p<0.01$) (Figure 2).

Influence of TGF-β1 on Myocardial Cell Apoptosis in Acute MI Rats

To explore the influence of TGF-β1 on myocardial cell apoptosis in acute MI rats, the myocardial cells in the model group were treated with TGF-β1. The influences of TGF-β1 on the cell pro-proliferation-related protein Bcl-2 and pro-apoptosis-related protein Bax were investigated. After treatment with TGF-β1, the expression level of Bax significantly decreased, while no evident changes were observed in Bcl expression (Figures 3A and 3B). Decreased

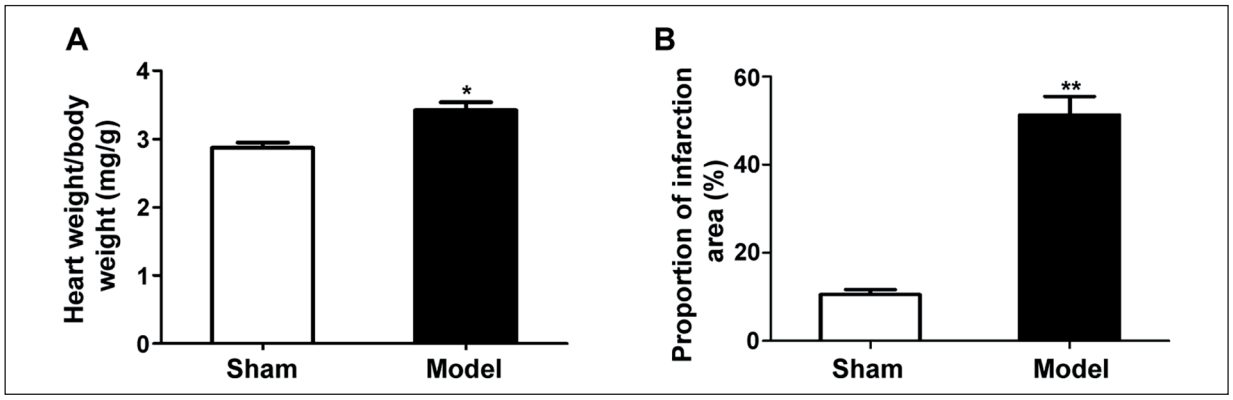

Figure 1. Heart weight/body weight ratio and infarction area. **A,** Heart weight/body weight (*$p<0.05$, the differences are significant), **B,** Proportion of infarction area (**$p<0.01$, the differences are extremely significant).

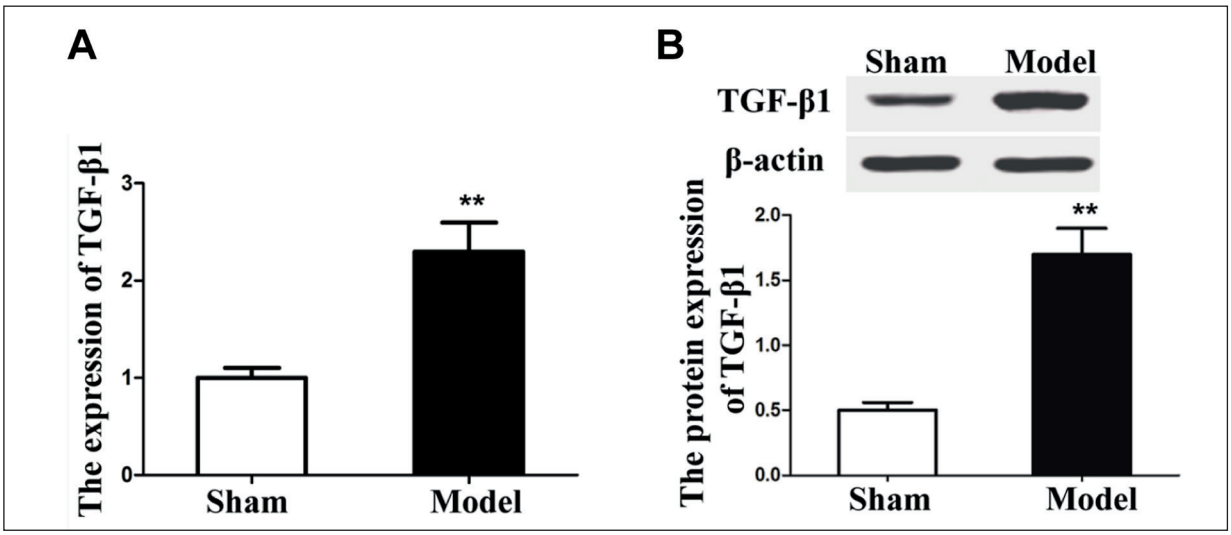

Figure 2. Expression of TGF-β1 in myocardial cells in the acute MI rat model. **A,** MRNA expression of TGF-β1 in myocardial cells (**p<0.01, the differences are extremely significant), **B,** Protein expression of TGF-β1 in myocardial cells (**p<0.01, the differences are extremely significant).

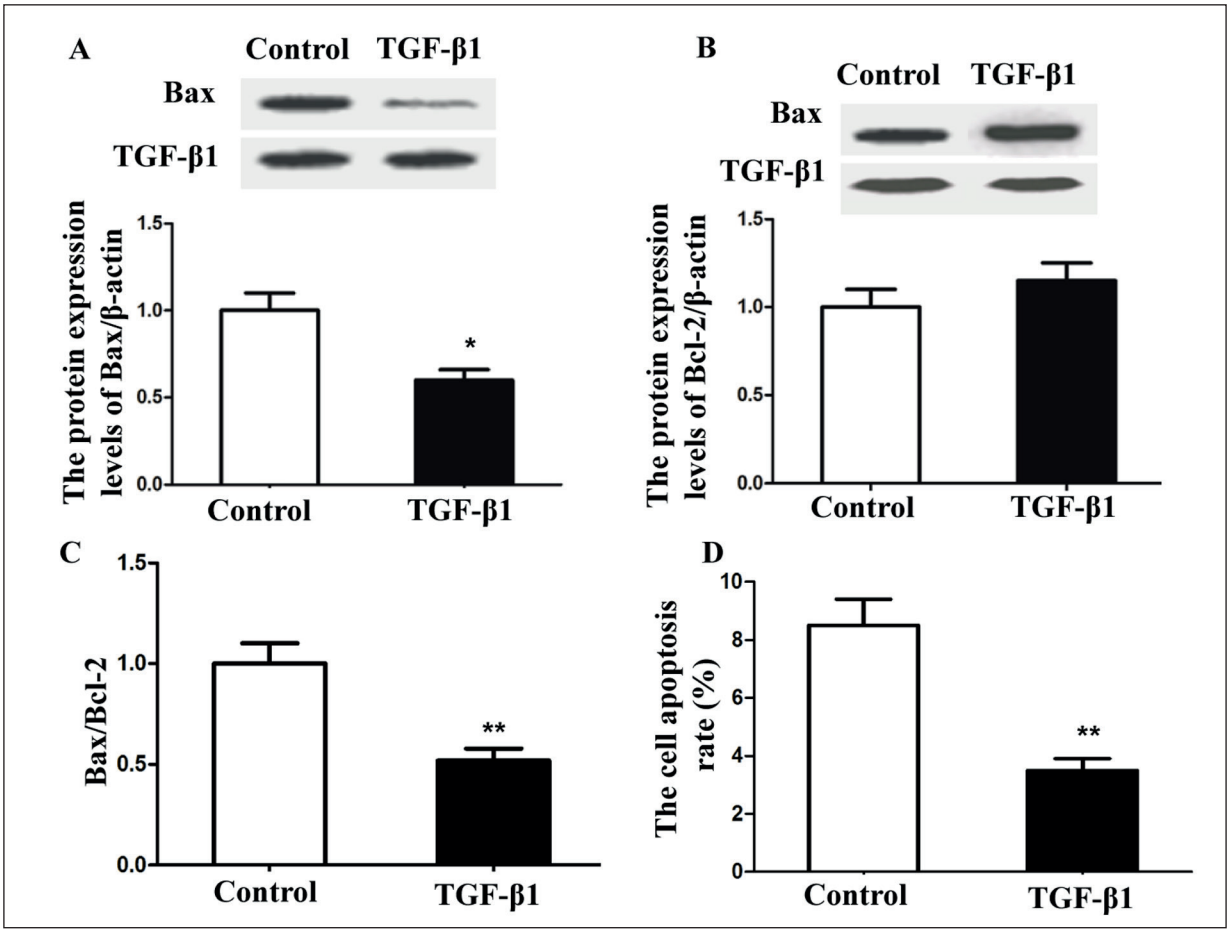

Figure 3. Influence of TGF-β1 on myocardial cell apoptosis in acute MI rats. **A, B,** Expression levels of Bax and Bcl-2 after treatment with TGF-β1 (*p<0.05, the differences are significant), **C,** Ratio of the expression level of Bax to that of Bcl-2 (**p<0.01, the differences are extremely significant.), **D,** Cell apoptosis detected via flow cytometry (**p<0.01, the differences are extremely significant).

Bax/Bcl-2 ratio indicated the influence of TGF-β1 on the proliferation and apoptosis of the cells treated with TGF-β1. Higher ratio indicated significantly stronger pro-apoptosis effect. Conversely, a lower ratio indicated stronger pro-proliferation effect. In the present study, the Bax/Bcl-2 ratio in the TGF-β1 group was notably lower than that of the control group (Figure 3C). Therefore, it could be speculated that the treatment with TGF-β1 exerted an anti-apoptotic effect on the myocardial cells in acute MI rats.

Flow cytometry results revealed that the treatment of TGF-β1 significantly decreased the proportion of apoptotic cells ($p<0.01$). The proportion of apoptotic cells in the control group and TGF-β1 group was (8.5 ± 0.9)% and (3.58 ± 0.4)%, respectively (Figure 3D).

TGF-β1 Affected Acute MI Rats via MAPK Signaling Pathway

Wu et al[13] have revealed that the MAPK signaling pathway is related to acute MI. Therefore, in the present study, we corroborated the changes in the MAPK signaling pathway in acute MI rats. Meanwhile, the protein expressions of p-MMK3/6 and MKK3/6 in myocardial cells in both model group and sham operation group were analyzed (Figures 4A and 4B). The results discovered that no significant changes were observed in the protein levels of MKK3/6 in the model group, while p-MKK3/6 was remarkably elevated. These results suggested that TGF-β1 activated MKK3/6 in the MAPK signaling pathway.

Discussion

Our results revealed that compared with sham operation group, the rats in the model group had significantly up-regulated mRNA and protein levels of TGF-β1 in the myocardium, as well as significantly elevated levels of p-MKK3/6. There seemed to be a cascade effect between these increases. Our results suggested that the TGF-β1/MAPK signaling pathway in myocardial cells in the MI region was activated during acute MI.

TGF-β1, a locally produced cytokine, has been considered as a leading cause of tissue fibrosis in various organic systems[14]. The research on acute MI and pressure overload-induced MI models has shown that the expression of TGF-β1 is up-regulated in the myocardium, suggesting that TGF-β1 participates in myocardial cell hypertrophy and fibrosis[15]. Over-expression of TGF-β1 in transgenic mice causes interstitial fibrosis and hypertrophic growth to myocardial cells[16]. TGF-β1 is responsible for the up-regulation of fetal contractile proteins in cultured neo-myocardial cells, such as β-myosin heavy chain (β-MHC) and α-skeletal actin[17]. However, little is known about the signaling pathway in myocardial cells with TGF-β1-induced hypertrophy after acute MI. Previous studies[18] have proved that the activated MKK3/6/p38 MAPK signaling pathway induces the expression of genes encoding sarcomeric proteins, which may also cause sarcomeric tissues in the myocardial cells.

Chen et al[19] have demonstrated that TGF-β1 is able to activate canonical and non-canonical signaling pathways to protect from myocardial reperfusion injury after ischemia. TGF-β1 pro-

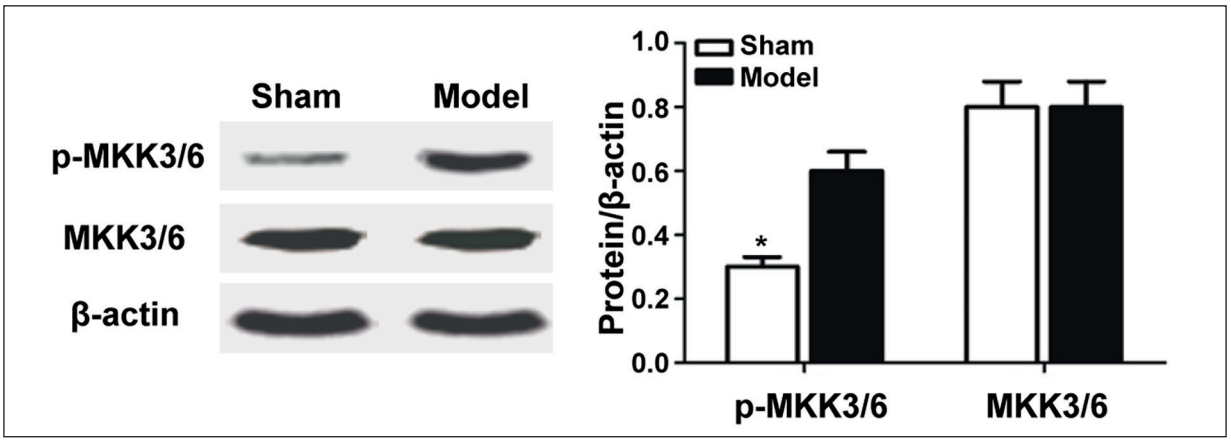

Figure 4. Protein expressions of MKK3/6 and p-MKK3/6 in myocardial cells of the two groups (*$p<0.05$, the differences are significant).

motes myocardial cell proliferation and differentiation into myofibroblasts[20]. At the heart level, TGF-β1 exerts anti-apoptosis effects *in vitro* and *in vivo*[21]. It has been proved that TGF-β1 is capable of decreasing or inactivating some key pro-apoptosis proteins, such as Bax and Caspase. However, such a growth factor can induce anti-apoptosis proteins, including Bcl-2[22]. In the present research, the treatment of TGF-β1 inhibited the apoptosis of myocardial cells in acute MI rats.

Zhang et al[12] have reported that after pressure overload, TGF-β-activated kinase 1 (TAK1) is activated in myocardial cells. In transgenic mice with activated TAK1, the ventricular hypertrophy impairs systolic and diastolic functions. Compared with control mice, the mRNA expression level of β-MHC in model mice increases by 20 times. In this study, significantly up-regulated mRNA level of β-MHC was observed in the acute MI rat model. Endogenous TAK1 may help to regulate the expression of β-MHC, thereby avoiding thickening myocardial wall after MI. Although initially separated as the target of TGF-β1, TAK1 can also be activated by interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α)[23]. Since the expressions of TNF-α and IL-1 are elevated in the left ventricle after MI, the TNF-α or IL-1 probably stimulates TAK1 in myocardial cells during acute MI. However, the expression levels of these cytokines have not been determined[24]. As one of the most important responses in cardiac remodeling, the activation of the TGF-β1/MAPK pathway involves the progression of heart failure. In this study, it was discovered that the level of p-MKK3/6 was significantly down-regulated in myocardial cells in the rats with acute MI. However, no evident change was observed in the total level of MKK3/6. In this sense, the above results are consistent with the literature indicating that in ischemic myocardial cells, the activities of MAPK and Akt are down-regulated[25]. However, another research has shown that acute MI can increase the activation levels of p-MKK3/6 and Akt in myocardial cells[25]. Peterson et al[26] have suggested that, with the inhibitor PD, the reperfusion aggravates injury in the heart of infarction rats.

Conclusions

In this study, we found that p-MKK3/6 could repress cell apoptosis by several mechanisms, including the inhibition of the capacity of the pro-apoptosis protein Bax. Furthermore, our findings suggested that the TGF-β1/MAPK signaling pathway might serve as a novel treatment target to inhibit ventricular remodeling after acute MI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Zhou M, Zou YG, Xue YZ, Wang XH, Gao H, Dong HW, Zhang Q. Long non-coding RNA H19 protects acute myocardial infarction through activating autophagy in mice. Eur Rev Med Pharmacol Sci 2018; 22: 5647-5651.
2) Moran AE, Tzong KY, Forouzanfar MH, Rothy GA, Mensah GA, Ezzati M, Murray CJ, Naghavi M. Variations in ischemic heart disease burden by age, country, and income: the global burden of diseases, injuries, and risk factors 2010 study. Glob Heart 2014; 9: 91-99.
3) Saeed O, Gupta V, Dhawan N, Streja L, Shin JS, Ku M, Bhoi S, Verma S. Knowledge of modifiable risk factors of Coronary Atherosclerotic Heart Disease (CASHD) among a sample in India. BMC Int Health Hum Rights 2009; 9: 2.
4) Schmitt J, Duray G, Gersh BJ, Hohnloser SH. Atrial fibrillation in acute myocardial infarction: a systematic review of the incidence, clinical features and prognostic implications. Eur Heart J 2009; 30: 1038-1045.
5) Duivenvoorden WC, Hirte HW, Singh G. Transforming growth factor beta1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. Clin Exp Metastasis 1999; 17: 27-34.
6) Nagahara H, Yamamoto A, Seno T, Obayashi H, Kida T, Nakabayashi A, Kukida Y, Fujioka K, Fujii W, Murakami K, Kohno M, Kawahito Y. Allograft inflammatory factor-1 in the pathogenesis of bleomycin-induced acute lung injury. Biosci Trends 2016; 10: 47-53.
7) Andonegui G, Ni A, Leger C, Kelly MM, Wong JF, Jalloul A, Winston BW. Sequential expression of IGF-IB followed by active TGF-beta1 induces synergistic pulmonary fibroproliferation in vivo. Am J Physiol Lung Cell Mol Physiol 2012; 303: L788-L798.
8) Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Koteliansky VE, Matthay MA, Sheppard D. TGF-beta is a critical mediator of acute lung injury. J Clin Invest 2001; 107: 1537-1544.
9) Takimoto Y, Aoyama T, Tanaka K, Keyamura R, Yui Y, Sasayama S. Augmented expression of neuronal

nitric oxide synthase in the atria parasympathetically decreases heart rate during acute myocardial infarction in rats. Circulation 2002; 105: 490-496.

10) Wunsch M, Sharma HS, Markert T, Bernotat-Danielowski S, Schott RJ, Kremer P, Bleese N, Schaper W. In situ localization of transforming growth factor beta 1 in porcine heart: enhanced expression after chronic coronary artery constriction. J Mol Cell Cardiol 1991; 23: 1051-1062.

11) Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. Cardiovasc Res 2004; 63: 423-432.

12) Zhang D, Gaussin V, Taffet GE, Belaguli NS, Yamada M, Schwartz RJ, Michael LH, Overbeek PA, Schneider MD. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. Nat Med 2000; 6: 556-563.

13) Wu Q, Xu H, Hao L, Ma G, Sun J, Song X, Ding F, Wang N. P2X7 receptor regulates sympathoexcitatory response in myocardial infarction rats via NF-κB and MAPK pathways. Am J Transl Res 2017; 9: 4954-4962.

14) Kim KK, Sheppard D, Chapman HA. TGF-beta1 signaling and tissue fibrosis. Cold Spring Harb Perspect Biol 2018; 10. pii: a022293.

15) Takahashi N, Calderone A, Izzo NJ, Maki TM, Marsh JD, Colucci WS. Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. J Clin Invest 1994; 94: 1470-1476.

16) Rosenkranz S, Flesch M, Amann K, Haeuseler C, Kilter H, Seeland U, Schlüter KD, Böhm M. Alterations of beta-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-beta(1). Am J Physiol Heart Circ Physiol 2002; 283: H1253-H1262.

17) Parker TG, Packer SE, Schneider MD. Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. J Clin Invest 1990; 85: 507-514.

18) Zechner D, Thuerauf DJ, Hanford DS, McDonough PM, Glembotski CC. A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression. J Cell Biol 1997; 139: 115-127.

19) Chen H, Li D, Saldeen T, Mehta JL. TGF-beta 1 attenuates myocardial ischemia-reperfusion injury via inhibition of upregulation of MMP-1. Am J Physiol Heart Circ Physiol 2003; 284: H1612-H1617.

20) Brønnum H, Eskildsen T, Andersen DC, Schneider M, Sheikh SP. IL-1β suppresses TGF-β-mediated myofibroblast differentiation in cardiac fibroblasts. Growth Factors 2013; 31: 81-89.

21) Bilodeau M, Tousignant J, Ethier C, Rocheleau B, Raymond VA, Lapointe R. Anti-apoptotic effect of insulin on normal hepatocytes in vitro and in vivo. Apoptosis 2004; 9: 609-617.

22) Surachetpong S, Jiranantasak T, Rungsipipat A, Orton EC. Apoptosis and abundance of Bcl-2 family and transforming growth factor beta1 signaling proteins in canine myxomatous mitral valves. J Vet Cardiol 2013; 15: 171-180.

23) Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995; 270: 2008-2011.

24) Sakurai H, Miyoshi H, Toriumi W, Sugita T. Functional interactions of transforming growth factor beta-activated kinase 1 with IkappaB kinases to stimulate NF-kappaB activation. J Biol Chem 1999; 274: 10641-10648.

25) Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Cytokine gene expression after myocardial infarction in rat hearts: possible implication in left ventricular remodeling. Circulation 1998; 98: 149-156.

26) Peterson JT, Li H, Dillon L, Bryant JW. Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res 2000; 46: 307-315.