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TGFβ1 protects myocardium from apoptosis and oxidative damage after ischemia reperfusion

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Abstract. – OBJECTIVE: Myocardial apoptosis is an important pathologic basis of ischemia-reperfusion injury (I/R). Transforming growth factor β1 (TGFβ1) participates in the regulation of oxidative damage and apoptosis. TGFβ1 is upregulated in the repair process of I/R injury. It is speculated that TGFβ1 over-expression is involved in the endogenous protective mechanism of I/R injury. This study explores the significance of TGFβ1 in myocardial cell apoptosis after I/R.

MATERIALS AND METHODS: Rat myocardial I/R injury model was established. Left ventricular ejection fraction (LVEF) and Left ventricular fractional shortening (LVFS) were detected by ultrasonic cardiogram. TGFβ1 expression in the myocardium was tested. H9C2 cells were cultured under ischemic hypoxic condition for 6 h, and then were treated by reoxygenation for 6 h to simulate I/R model. H9C2 cells were divided into three groups, including I/R+pIRES2-Blank, I/R+pIRES2 TGFβ1, and I/R+pIRES2-TGFβ1+LY364947. TGFβ1 mRNA and protein levels were evaluated. Cell apoptosis and reactive oxygen species (ROS) were determined by flow cytometry.

RESULTS: LVEF and LVFS significantly decreased in I/R group compared with Sham group. TGFβ1 mRNA and protein expressions in myocardium from I/R group up-regulated than the control. I/R treatment markedly elevated TGFβ1 mRNA and protein levels, increased ROS content, and enhanced cell apoptosis in H9C2 cells. Over-expression of TGFβ1 significantly weakened ROS production and apoptosis in H9C2 cells after I/R. TGFβ receptor inhibitor LY364947 restrained ROS production and apoptosis attenuation in H9C2 cells treated by TGFβ.

CONCLUSIONS: TGFβ1 alleviates myocardial cell apoptosis after I/R. Blocking TGFβ1 attenuates the protective effect of TGFβ1 on I/R injury.

Key Words:
TGFβ1, I/R, Myocardial cell, Apoptosis, ROS.

Introduction

Acute myocardial infarction (AMI) refers to myocardial necrosis caused by the acute and persistent ischemia-hypoxia of the coronary artery. In the clinic, it is mainly presented as fierce and permanent retrosternal pain, which can be complicated with cardiac arrhythmia, shock, or heart failure[1]. Coronary artery reperfusion after AMI is the most effective method to save ischemic myocardium, protect heart function, and rescue patient life[2]. At present, the most efficient way on AMI is to recover the blood supply in the myocardium in the clinic, thus to achieve the reperfusion. The commonly used methods include intravenous coronary thrombolysis (IVCT), coronary artery bypass grafting (CABG), and percutaneous transluminal coronary angioplasty (PTCA), which can achieve the goal of saving ischemic myocardium and rescuing the patient life via restoring the blood supply of the ischemic myocardium after AMI[3]. However, blood reperfusion inevitably induces ischemia-reperfusion (I/R) injury, seriously restricting the clinical curative effect[4]. Myocardial cell apoptosis and necrosis are the main pathophysiological processes of I/R injury. Cell apoptosis occurs earlier and almost sustains throughout the whole process of I/R injury, thus is also the main form of myocardial cell injury[5]. I/R injury may aggravate the structure and fun-

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ction of ischemic myocardium after AMI, resulting in deterioration of ventricular remodeling, cardiac function deterioration, and continuous cardiac dysfunction after AMI[6]. Transforming growth factor β1 (TGFβ1) is a kind of protein peptide with a variety of functions, such as regulating cell growth[7], differentiation, apoptosis[8], and anti-oxidation[9]. TGFβ1 is found to be upregulated in the I/R injury repair of heart, brain, kidney, intestine, and other organs, suggesting that TGFβ1 elevation may be involved in the endogenous protection of I/R injury[10-13]. Therefore, the over-expression of TGFβ1 may protect the organ from I/R injury. This study discussed the significance of TGFβ in inducing myocardial cell apoptosis in myocardial ischemia-reperfusion model.

Materials and Methods

Materials and Reagents

Healthy adult male Wistar rats at 6-8 weeks old and 220-250 g weighted were purchased from Three Gorges University (Hubei, China). Rat myocardial cell line H9C2 was bought from ScienCell Co. Ltd. (Carlsbad, CA, USA). DMEM medium was got from Hyclone (Logan, UT, USA). FBS was bought from Biological Industries (Beit Haemek, Israel). Trizol was obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). PrimeScript™ RT reagent Kit and SYBR Green were purchased from TaKaRa (Dalian, China). Rabbit anti TGFβ1 antibody was got from Abcam Biotech (Catalogue No. ab92486; 1:3000; Cambridge, MA, USA). Mouse anti β-actin antibody was bought from Cell Signaling Technology (Catalogue No. 3700; 1:2000; Beverly, MA, USA). TUNEL apoptosis detection kit, Annexin V-FITC/PI kit, and DCFH-DA probe were obtained from Beyotime (Beijing, China). The horse radish peroxidase (HRP) labeled secondary antibody was bought from Jackson ImmunoResearch (West Grove, PA, USA). TGFβ receptor inhibitor LY364947 was from MedChemExpress (Princeton, NJ, USA).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Three Gorges University People's Hospital.

Rat I/R Model Establishment

The rat was anesthetized by chloral hydrate intraperitoneal injection. The limbs were connected to the electrocardiogram, and the endotracheal intubation was connected animal breathing machine. The chest was opened on the left 4th intercostal space, and the left anterior descending coronary artery was identified between pulmonary arterial cone and aorta. Next, the artery was ligated with 6-0 suture. AMI modeling success was judged as ST segment arch lift 0.1 mV on Q lead or T wave high amplitude, myocardial color wanning, and pulse weakened. The blood supply was restored after blocking for 60 min. Sham group was selected as control. The rats in the test group were divided into three subgroups with six in each subgroup, including postoperative 6 h, 12 h, and 24 h, respectively.

TUNEL Assay

The rats were killed at postoperative 6 h, 12 h, and 24 h. The myocardium tissue was collected to prepare frozen section. The section was fixed in 4% paraformaldehyde at room temperature for 60 min and treated by 0.1% Triton X-100 on ice for 2 min. Next, the section was washed by phosphate buffered saline (PBS) for twice and incubated in 50 μl TUNEL at 37°C for 60 min. At last, the section was observed under the fluorescence microscope.

Ultrasonic Cardiogram

The rats were anesthetized by 10% chloral hydrate at 24 h after operation. Ultrasonic cardiogram was performed through the chest. The left ventricular end systolic and diastolic diameters were recorded through the anterior papillary muscle level of the mitral valve. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were automatically calculated.

H9C2 Cell Transfection and I/R Treatment

Rat myocardial cells H9C2 were routinely cultured in DMEM medium containing 10% fetal bovine serum (FBS) and maintained at 37°C and 5% CO2. The cells in logarithmic phase were transfected by TGFβ1 over-expression vector pIRES2-TGFβ1 or empty vector Pires2-Blank. The cells were further treated by I/R at 72 h after transfection. For I/R treatment, the cells were cultured in low glucose serum free DMEM to simulate ischemic condition. Next, the cells were maintained in an incubator with 5% CO2 and 95% N2 to simulate hypoxic condition. The cells were changed to routine medium after 6 h and further cultured

in normal condition for 6 h. H9C2 cells were divided into three groups, including I/R+pIRES2-Blank, I/R+pIRES2 TGFβ1, and I/R+pIRES2-TGFβ1+LY364947. LY364947 was used to treat cells after transfection at 50 nmol/L.

Cell Apoptosis Detection

The cells were digested by the enzyme and washed by precooled PBS. Then, the cells were suspended in 500 μl Binding Buffer and incubated in 5 μl Annexin V-FITC avoid of light at room temperature for 15 min. Next, the cells were stained by 5 μl PI and tested on flow cytometry.

DCFH-DA Probe Detection of ROS Content

DCFH-DA was diluted by serum free medium at 1:1000 to make the final concentration at 10 μmol/L. The cells were added with DCFH-DA and incubated at 37°C for 20 min. After washed with serum free medium for three times, the cells were resuspended in 500 μl PBS and tested on flow cytometry at 488 nm.

qRT-PCR

Total RNA was extracted using Trizol and reverse transcribed to cDNA using PrimeScript™ RT reagent Kit. The cDNA was used as the template for PCR amplification. The primers used were as follows. TGFβ1PF:5′-TGAGTGGCTGTCTTTTGACG-3′, TGFβ1PR:5′-ACTTCCAACCCAGGTCCTTC-3′; β-actinPF:5′-TGGGACGATATGGAGAAGAT-3′,β-actinPR:5′-ATTGCCGATAGTGATGACCT-3′. The total reaction system contained 5 μl 2×SYBR Green Mixture 5.0 μL, 0.5 μl positive and reverse primers, 1 μl cDNA, and ddH2O. The reaction was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on ABI ViiA7.

Western Blot

Total protein was extracted and separated by SDS-PAGE. Then the protein was transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, New Jersey, USA) and blocked with 5% skim milk at room temperature for 60 min. Next, the membrane was incubated with primary antibody (TGFβ1 at 1:3200, β-actin at 1:500) at 4°C overnight and secondary antibody at 1:5000 at room temperature for 60 min. At last, the membrane was developed by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) and scanned.

Statistical Analysis

All data analyses were performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and compared by t-test. Between-group difference was tested by using Tukey's post hoc test. $p<0.05$ was considered as statistical significance.

Results

Cardiac Function Reduced, while Myocardial Cell Apoptosis Enhanced After I/R Injury

Echocardiography showed that LVFS exhibited no changes in Sham group, while it was significantly decreased in I/R group with time dependence (Figure 1A). LVEF showed no statistical changes in Sham group, whereas it obviously declined in I/R group with time dependence (Figure 1B). Flow cytometry revealed that myocardial cell apoptosis rate at each time point in I/R group was markedly higher than that in Sham group (Figure 1C). It demonstrated that I/R weakened myocardial function and induced cell apoptosis.

TGFβ1 Unregulated in Myocardium After I/R

qRT-PCR showed that TGFβ1 mRNA expression significantly elevated in myocardium from I/R tissue compared with Sham group (Figure 2A). Western blot revealed that TGFβ1 protein level obviously enhanced in I/R group compared with Sham (Figure 2B).

I/R Treatment Promoted TGFβ1 Expression and Apoptosis of H9C2 Cells

Compared with control, I/R treatment markedly increased TGFβ1 mRNA and protein expressions in H9C2 cells (Figure 3A and B). Flow cytometry detection demonstrated that I/R treatment induced ROS production in H9C2 cells (Figure 3C). Moreover, TUNEL assay showed that I/R treatment elevated H9C2 cell apoptosis (Figure 3D).

TGFβ1 Overexpression Alleviated ROS Production and Apoptosis of H9C2 Cells After I/R

Compared with H9C2 cells transfected by pIRES2-Blank, TGFβ1 protein significantly increased in H9C2 cells transfected by pIRES2-TGFβ1, suggesting successful TGFβ1 over-expression (Figure 4A). Flow cytometry revealed that H9C2

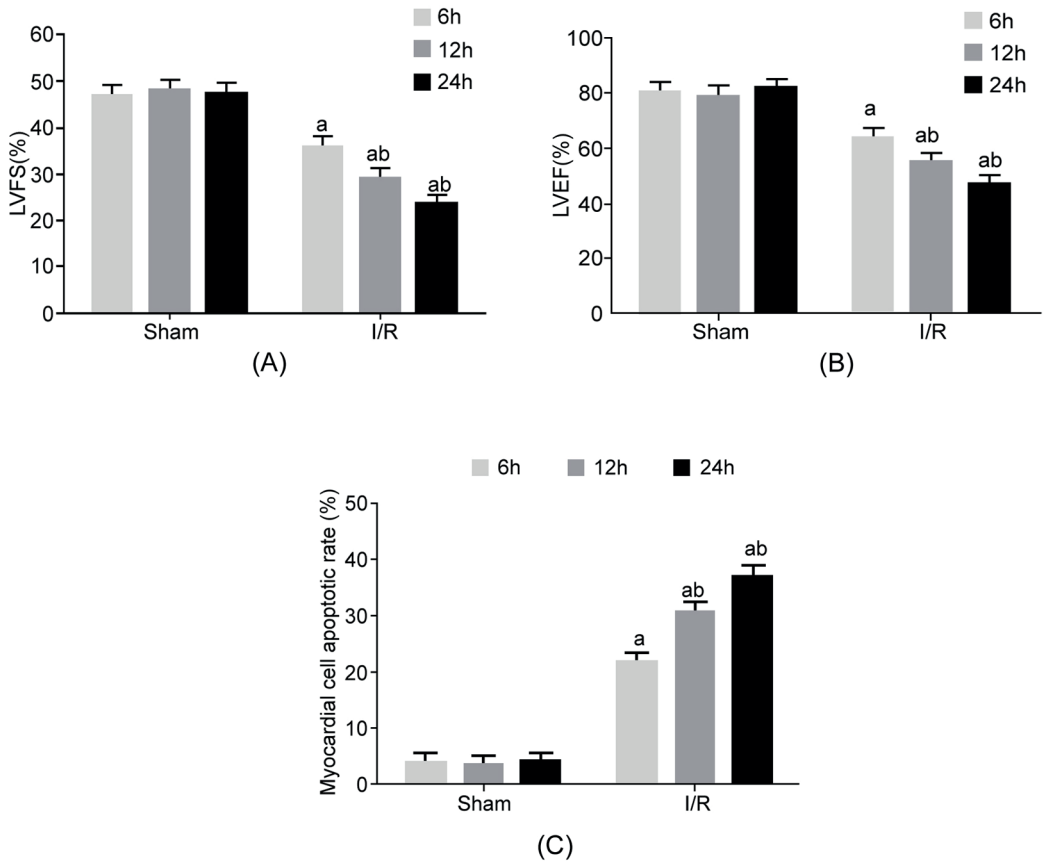

Figure 1. Cardiac function reduced, while myocardial cell apoptosis enhanced after I/R injury. ***(A)*** ultrasonic cardiogram detection of LVFS; ***(B)*** ultrasonic cardiogram detection of LVEF; ***(C)*** TUNEL assay detection of cell apoptosis. a $p<0.05$, compared with Sham group. b, $p<0.05$, compared with 6 h.

cell apoptosis rate after I/R was obviously declined after transfected by TGFβ1 plasmid, while LY364947 treatment increased cell apoptosis (Figure 4B). DCFH-DA probe staining demonstrated that TGFβ1 over-expression markedly reduced ROS production in H9C2 cells after I/R, whereas LY364947 attenuated ROS reduction (Figure 4C).

Discussion

AMI is a common cardiovascular disease that is one of the most usual causes of heart failure[14]. AMI is characterized as a variety of inducements, complicated pathogenesis, and in critical condition. Timely and effectively treatment is the key to save the life and reduce the complications[15]. Following the increase of elderly population proportion, speeding up of life rhythm, diet change, and the enhancement of psychological pressure, the incidence of AMI keeps rising in our country. AMI caused by ischemia hypoxia is usually treated by thrombolysis, coronary artery dilatation, and coronary artery bypass surgery in the clinic to restore the blood reperfusion. However, myocardial I/R does not make the myocardium back to the normal physiological environment, but causes more serious I/R injury at the same time[16]. Therefore, exploration of the mechanism of I/R injury to restore blood supply and reduce the I/R injury is of great significance to improve AMI treatment effect and prognosis[17]. Cell apoptosis is the characteristic change of I/R injury, which determines the degree of I/R injury. Also, the degree of myocardial cell apoptosis affects the AMI treatment effects and the prognosis. Myocardial apoptosis plays an important role in the process of cardiac remodeling pathological physiology after I/R. The attenuation of myocardial cell apoptosis can improve myocardial function after ischemia and delay myocardial remodeling process[18].

TGFβ is a kind of superfamily protein polypeptide with similar structure and hormone activity. Up to now, there are more than 30 kinds of TGFβ

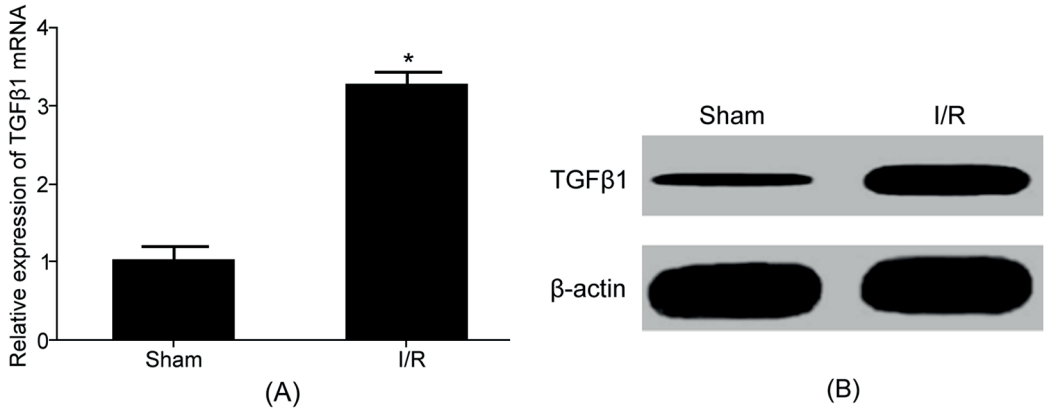

Figure 2. TGFβ1 uregulated in myocardium after I/R. ***(A)*** qRT-PCR detection of TGFβ1 mRNA expression; ***(B)*** Western blot detection of TGFβ1 protein expression. a $p<0.05$, compared with Sham group.

protein were found[19]. Almost all normal and cancerous tissues and cells can express some TGFβ and TGFβ receptor. TGFβ superfamily contained at least 6 kinds of TGFβ, while three types were identified from mammalian, including TGFβ1, TGFβ2, and TGFβ3. TGFβ1 showed the highest content and strongest activity[8]. TGFβ1 is a type of protein peptide with multiple functions. It involves in cell proliferation[7], differentiation, apoptosis[8], anti-oxidation damage[9], and other biological

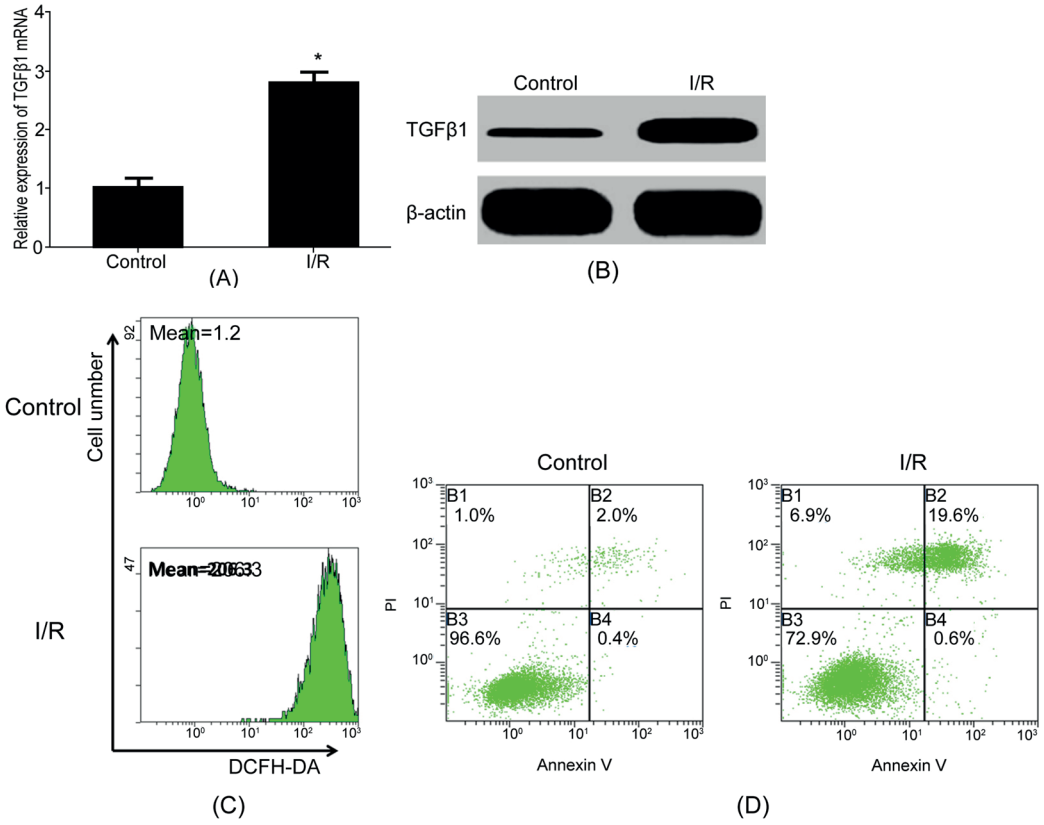

Figure 3. I/R treatment promoted TGFβ1 expression and apoptosis of H9C2 cells. (A) qRT-PCR detection of TGFβ1 mRNA expression; (B) Western blot detection of TGFβ1 protein expression; (C) Flow cytometry detection of ROS; (D) Flow cytometry detection of cell apoptosis. * $p<0.05$, compared with control.

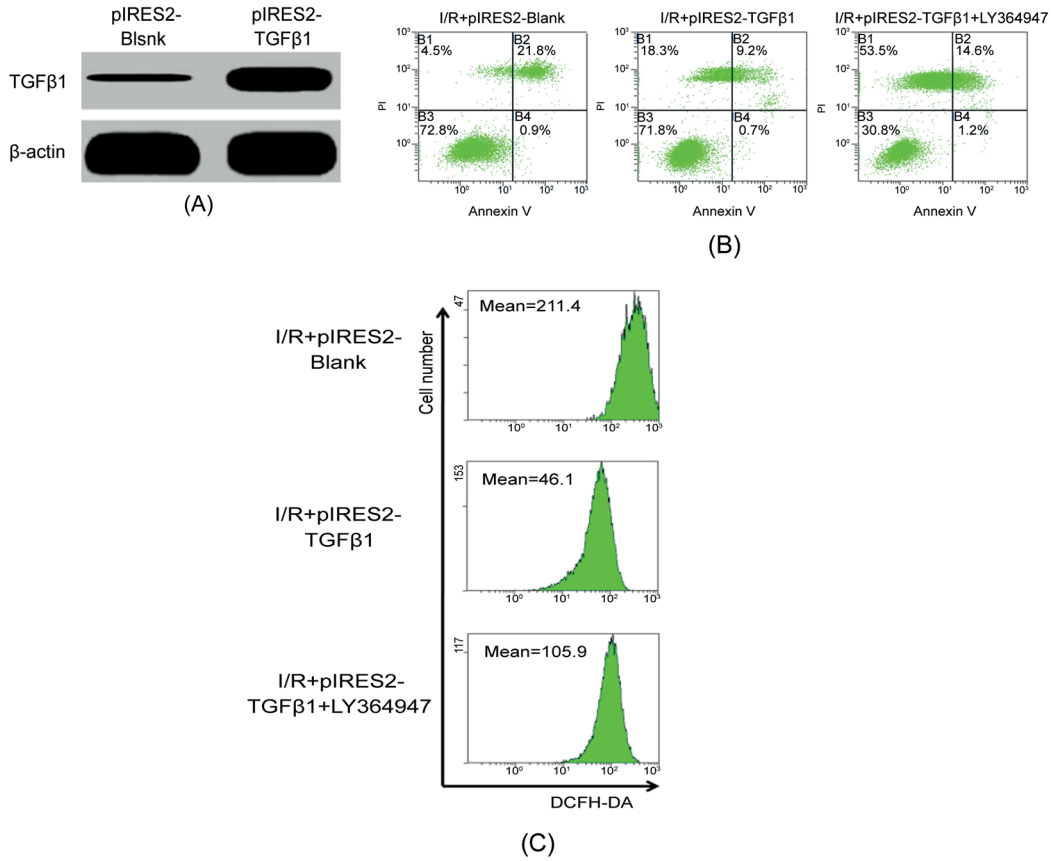

Figure 4. TGFβ1 overexpression alleviated ROS production and apoptosis of H9C2 cells after I/R. ***(A)*** Western blot detection of TGFβ1 protein expression; ***(B)*** Flow cytometry detection of cell apoptosis; ***(C)*** Flow cytometry detection of ROS content.

processes. Numerous studies[10-13] demonstrated that TGFβ1 upregulated in heart, brain, and kidney during I/R. TGFβ1 changes during I/R injury suggested it may be involved in the repair process of injury. On the contrary, endogenous TGFβ1 deficiency may be related to or aggravate I/R injury[10,11]. TGFβ1 is mainly produced by myocardial cells and cardiac fibroblasts. It was found that TGFβ1 expressive abundance was low in the normal myocardium, while it exhibited certain elevation during ischemia hypoxia or I/R[10,11]. It is speculated that TGFβ1 upregulation may participate in the endogenous protective mechanism of the myocardial cell I/R injury. Thus, increasing TGFβ1 expression may alleviate I/R injury. This study explored the significance of TGFβ in myocardial cell apoptosis induced by I/R.

Echocardiography showed that LVFS and LVEF exhibited no changes in Sham group, while they were significantly decreased in I/R group with time dependence. It indicated the successful establishment of rat I/R model. Flow cytometry revealed that myocardial cell apoptosis rate at each time point in I/R group was markedly higher than that in Sham group, which was similar to the report of Jian et al[20] and Zhang et al[21]. Our findings demonstrated that TGFβ1 expression in myocardium from I/R rat was markedly higher than the Sham group. Mortazavi-Haghighat et al[22] reported that I/R treatment apparently elevated TGFβ1 expression in a blood vessel and skin fibroblast. Yang et al[10] and Chen et al[11] showed that TGFβ1 increased in the myocardium during I/R injury. Our study was similar to Mortazavi-Haghighat et al[22], Yang et al[10], and Chen et al[11]. Nevertheless, I/R treatment significantly upregulated TGFβ1 level in H9C2 cells cultured *in vitro*. It also suggested that TGFβ1 elevation may be involved in the endogenous protective mechanism of myocardial cells during I/R injury. Further analysis exhibited that TGFβ1 overexpression significantly alleviated ROS production and apoptosis of H9C2 cells after I/R injury. TGFβ receptor inhibitor LY364947 reduced the declination

of ROS production and cell apoptosis, indicating that enhancing TGFβ and suppressing TGFβ activity may alleviate and aggravate myocardial cell injury after I/R, respectively. Chen et al[11] showed that TGFβ1 pretreatment can significantly decline MMP-1 production in myocardial cells after I/R and attenuate I/R induced myocardial cell injury. Grunenfelder et al[23] demonstrated that TGFβ1 over-expression can reduce I/R injury of donor's heart in heart transplantation. Vivar et al[24] reported that I/R treatment-induced cardiac fibroblasts apoptosis, while TGFβ1 pretreatment obviously declined cell apoptosis induced by I/R. Its specific mechanism of apoptosis inhibition was based on activation of canonical TGFβ/Smad3 and noncanonical TGFβ/EKR/Akt signaling pathways. Kim et al[25] suggested that the application of neutralizing antibody to block the bioactivity of TGFβ1 apparently enhanced I/R treatment induced intestinal membrane injury. Lee et al[26] showed that sevoflurane can protect I/R renal injury through TGFβ1, whereas blocking TGFβ1 weakened the protective effect of sevoflurane. All of these studies confirmed the protective role of TGFβ1 on I/R induced injury, which was in accordance with our results. Specifically, TGFβ1 may reduce cell apoptosis via upregulating anti-apoptotic gene Bcl-2 expression[10,23,27], decline nitric oxide synthase translation, NO synthesis, and decrease nitrogenous free radical production[28,29]. This study indicated that TGFβ1 exhibited protective role on myocardial cell apoptosis induced by I/R injury, while the specific mechanism was still unclear. Further in-depth investigation is needed.

Conclusions

We observed that TGFβ1 alleviates myocardial cell apoptosis after I/R. Blocking TGFβ1 attenuates the protective effect of TGFβ1 on I/R injury.

Conflict of interest

The authors declare no conflicts of interest.

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