

TGF- β 1 induces apoptosis of bone marrow-derived mesenchymal stem cells via regulation of mitochondrial reactive oxygen species production

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Abstract. Bone marrow-derived mesenchymal stem cells (BMSCs) are the most promising seed cells in regenerative medicine. Our previous study demonstrated that transforming growth factor (TGF)- β 1 induced BMSC senescence *in vitro*. Whether TGF- β 1 affects the apoptosis of BMSCs has not been examined; therefore the aim of the present study was to investigate this effect. BMSCs were isolated from mouse bone marrow, and the third-passage cells were exposed to 0, 10 and 20 ng/ml TGF- β 1 for 24 h. Cell proliferation was measured by MTT assay; apoptosis was assessed using DAPI staining; and the apoptotic signals Annexin V, B-cell lymphoma (Bcl)-2 and Bcl-2-associated X protein (Bax) were measured using western blotting. Mitochondrial reactive oxygen species (ROS) were measured by flow cytometry following staining with MitoSOXTM Red mitochondrial superoxide indicator. The MTT assay showed that 10 and 20 ng/ml TGF- β 1 inhibited BMSC proliferation. DAPI staining demonstrated that 10 and 20 ng/ml TGF- β 1 promoted BMSC apoptosis, which was further confirmed by a western blotting assay showing a significant increase in the pro-apoptotic signals Annexin V and Bax but a decrease in the anti-apoptotic signal Bcl-2. It was also found that TGF- β 1 markedly increased the mitochondrial ROS levels in BMSCs. It is well known that mitochondrial ROS are strong stimulators of cell apoptosis. These findings indicate that TGF- β 1 can induce BMSC apoptosis, and the mechanism may involve mitochondrial ROS generation.

Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stromal cells with the potential to differentiate into a variety of cell types, including adipocytes, osteocytes, cardiomyocytes and neurons (1). Recently, BMSCs have been used to treat acute and chronic myocardial infarction. Transplantation of BMSCs into the left ventricle has been demonstrated to decrease the infarcted area, inhibit left ventricular remodeling and improve cardiac function in patients with myocardial infarction or animal models with heart ischemia (2-5). Generally, BMSCs need to be induced *in vitro* by special reagents for cardiomyogenic differentiation. Transforming growth factor- β 1 (TGF- β 1) is one of the most commonly used reagents for inducing the cardiomyogenic differentiation of BMSCs (6-8). Previous studies have indicated that TGF- β 1 treatment *in vitro* promotes the transformation of BMSCs into cardiomyocyte-like cells that express cardiomyocyte-specific markers (6-8).

TGF- β 1 is a growth factor with multiple functions. It not only affects cell differentiation but also plays diverse roles in regulating cell proliferation, migration, apoptosis and autophagy (9-11). Previous studies have indicated that TGF- β 1 inhibits the proliferation of epithelial cells, cardiac fibroblasts, hematopoietic cells and cancer cells but promotes the proliferation of vascular smooth muscle cells, skin fibroblasts, endothelial cells and other specialized cells (12-14). TGF- β 1 has also been demonstrated to induce the apoptosis of a wide range of cells (15-17). Mitochondrial reactive oxygen species (ROS) are a family of reactive molecules that can trigger cell apoptosis by damaging mitochondrial DNA (18). It is known that TGF- β 1 can induce mitochondrial ROS production in numerous cell lineages (19). Our previous study showed that TGF- β 1 induced BMSC senescence by promoting mitochondrial ROS generation (20); however, whether TGF- β 1 induces the apoptosis of BMSCs through the regulation of mitochondrial ROS production has not been elucidated, and this topic is a focus of the present study.

Materials and methods

Animals. A total of 6 male C57BL/6 mice (8 weeks old) were obtained from the Experimental Animal Center of Xinxiang

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Medical University (Xinxiang, China). The study protocol and animal use were approved by the Ethics Committee of Xinxiang Medical University. Animal treatment was strictly performed according to the Guidelines of the Ministry of Science and Technology of the People's Republic of China (approval no. 2006-398).

Isolation and culture of BMSCs. BMSCs were isolated and cultured according to a recently published protocol (21). The third-passage BMSCs were exposed to 0, 10 and 20 ng/ml recombinant mouse TGF- β 1 (Cell Signaling Technology, Inc., Danvers, MA, USA) for 24 h, and cell proliferation, apoptosis and mitochondrial ROS were subsequently measured.

MTT assay. BMSC viability was measured with an MTT assay kit (American Type Culture Collection, Manassas, VA, USA) following exposure to different concentrations of TGF- β 1. The third-passage BMSCs (3×10^3 cells/well) were placed in 96-well plates and cultured with Dulbecco's modified Eagle's medium (DMEM) without serum overnight. The next day, the medium was replaced with complete DMEM (with 15% serum) supplemented with 0, 10 and 20 ng/ml TGF- β 1, and the cells were cultured for an additional 24 h. A total of 10 μ l MTT reagent was then added to each well and the plates were incubated at 37°C for 2 h. Following incubation, MTT detergent reagent (100 μ l) was added to each well and the plates were incubated at room temperature for 2 h in the dark. Finally, absorbance at 570 nm was read using a plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay. Cell apoptosis was measured using DAPI (Beyotime Institute of Biotechnology, Shanghai, China) staining. BMSCs (1×10^5 cells/well) were seeded in 24-well plates with round coverslips and exposed to 0, 10 and 20 ng/ml TGF- β 1 for 24 h. Following washing with phosphate-buffered saline (PBS), the cells were fixed with buffered paraformaldehyde and incubated with 0.1% TritonTM X-100 (Bao Biological Engineering Co., Ltd., Dalian, China) for 10 min at room temperature. The cells were treated with DNase-Free RNase (50 mg/ml) for 3 h at 37°C and then stained with 5 μ M DAPI for 5 min at room temperature. Cell apoptosis was assessed under a fluorescence microscope (Olympus Corp., Tokyo, Japan). The cells with a condensed nucleus or a nucleus with irregular edges were considered to be apoptotic.

Western blotting. Following treatment with different concentrations of TGF- β 1 for 24 h, proteins were extracted from the BMSCs using a protein extraction kit containing 20 ml 5X lysis buffer, 0.5 ml protease inhibitor, 0.5 ml phosphatase inhibitor and 0.5 ml phenylmethylsulfonyl fluoride (Bao Biological Engineering Co., Ltd.). Protein concentrations were measured using a standard Bradford protein assay method. Western blot analysis was performed according to a recently published protocol (22). In brief, 20 μ g proteins were mixed with equal volume of 2X loading buffer (Bao Biological Engineering Co., Ltd.) and heated at 95°C for 5 min. Proteins were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bao Biological Engineering Co., Ltd.), then transferred onto polyvinylidene fluoride membranes (Bao Biological Engineering Co., Ltd.). The membranes were

Table I. Rate of apoptosis in each group.

Group	Rate of apoptosis (%)
Control	5.164 \pm 1.437
10 ng/ml TGF- β 1	26.412 \pm 3.235 ^a
20 ng/ml TGF- β 1	38.857 \pm 4.286 ^a

Data are presented as the mean \pm standard deviation. ^aP<0.05 vs. control. TGF- β 1, transforming growth factor- β 1.

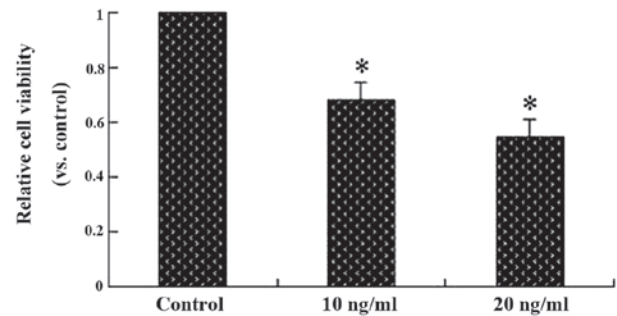


Figure 1. Cell viability of bone marrow-derived mesenchymal stem cells following exposure to 0, 10 and 20 ng/ml transforming growth factor- β 1 for 24 h. ^aP<0.05 vs. control.

blocked with 5% non-fat milk (Bao Biological Engineering Co., Ltd) in Tris-buffered saline with Tween-20 (TBS-T; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) and incubated with the following primary antibodies: Rabbit polyclonal IgG anti-B-cell lymphoma 2 (Bcl-2; 1:1,000; sc-492; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal IgG anti-Bcl-2-associated X protein (Bax; 1:1,000; sc-493; Santa Cruz Biotechnology, Inc.), rabbit polyclonal IgG anti-Annexin V (1:2,000; ab14196; Abcam, Cambridge, MA, USA) or mouse monoclonal IgG anti- β actin (1:2,000; sc-47778; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Following washing with TBS-T three times, the membranes were incubated with donkey anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:10,000; sc-2313; Santa Cruz Biotechnology, Inc.) or donkey anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (1:10,000; sc-2314; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The immunoreactive bands were visualized with an Enhanced Chemiluminescence Western Blotting Substrate Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The mean expression levels of Annexin V, Bax and Bcl-2 in each group were calculated using Image J software [National Institutes of Health (NIH), Bethesda, MD, USA].

Mitochondrial ROS measurement. Mitochondrial ROS were measured by flow cytometry following staining with MitoSOXTM Red mitochondrial superoxide indicator (Invitrogen Life Technologies). The BMSCs were plated in 24-well plates, treated with different concentrations of TGF- β 1 for 24 h and then detached using 2.5% trypsin digestion. Following digestion, the cells were suspended in warm PBS and incubated with 5 μ M MitoSOX reagent for 10 min at

Table II. Relative expression levels of Annexin V, Bax and Bcl-2 in each group.

Protein	Control	10 ng/ml TGF- β 1	20 ng/ml TGF- β 1
Annexin V (%)	58.262 \pm 4.372	74.972 \pm 6.301 ^a	89.331 \pm 6.996 ^a
Bax (%)	42.964 \pm 2.438	76.250 \pm 6.152 ^a	95.952 \pm 5.468 ^a
Bcl-2 (%)	41.194 \pm 3.875	26.412 \pm 3.235 ^a	10.513 \pm 3.781 ^a

Data are presented as the mean \pm standard deviation. ^aP<0.05 vs. control. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; TGF- β 1, transforming growth factor- β 1.

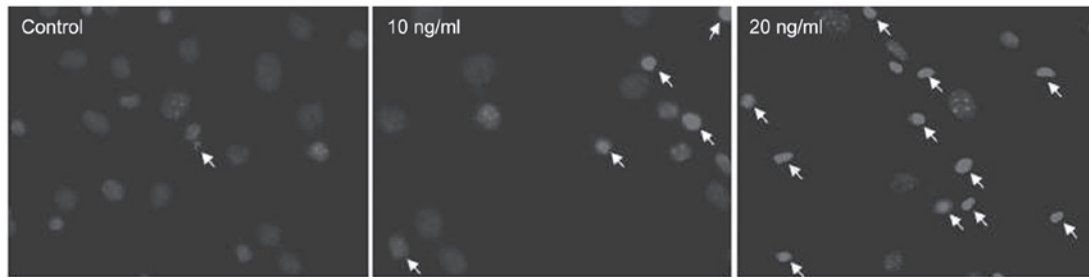


Figure 2. DAPI staining showing apoptosis of bone marrow-derived mesenchymal stem cells following exposure to 0, 10 and 20 ng/ml transforming growth factor- β 1 for 24 h. The arrows indicate apoptotic cells with condensed or irregular nuclei (magnification, x200). ^{*}P<0.05 vs. control.

37°C in the dark. The cells were then washed thrice with PBS, and the mitochondrial ROS levels were analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Statistical analysis. Statistical analysis was performed with SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA). Data are presented as the mean \pm standard deviation. Univariate comparisons of means were performed using one-way analysis of variance followed by Tukey tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of TGF- β 1 on BMSC viability. Compared with the control, treatment with 10 and 20 ng/ml TGF- β 1 for 24 h significantly decreased BMSC viability (Fig. 1).

Effect of TGF- β 1 on apoptosis of BMSCs. An increased number of cells with a condensed nucleus or a nucleus with irregular edges (DAPI staining) was observed in the TGF- β 1 (10 and 20 ng/ml)-treated cells compared with the control cells, which shows that TGF- β 1 can stimulate BMSC apoptosis (Fig. 2). The mean rate of apoptosis in each group was calculated, and the results are shown in Table I.

Effect of TGF- β 1 on the expression of apoptotic signals in BMSCs. Annexin V is an anticoagulant protein that is typically used as an indicator of cell apoptosis. Bcl-2 is an important member of the Bcl-2 family of regulator proteins that inhibits cell apoptosis (23). Bax is an antagonist of Bcl-2 that promotes cell apoptosis (23). Western blotting showed that treatment with 10 and 20 ng/ml TGF- β 1 significantly increased the expression of Annexin V and Bax but decreased the expression of Bcl-2 (Fig. 3). The average

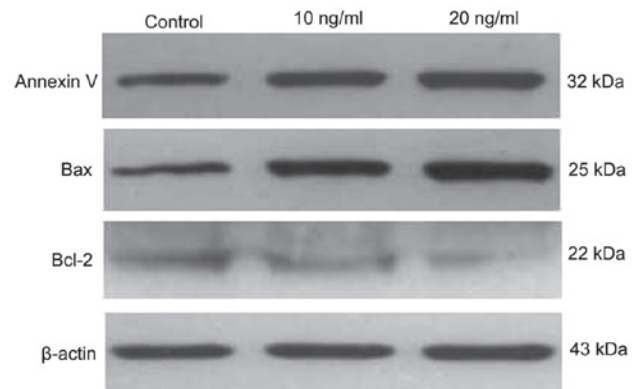


Figure 3. Western blotting showing the expression of Annexin V, Bax and Bcl-2 in bone marrow-derived mesenchymal stem cells following exposure to 0, 10 and 20 ng/ml transforming growth factor- β 1 for 24 h. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

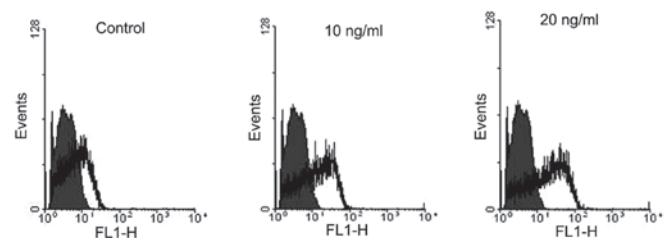


Figure 4. Flow cytometry showing the effect of transforming growth factor- β 1 on mitochondrial reactive oxygen species in bone marrow-derived mesenchymal stem cells.

expression levels of Annexin V, Bax and Bcl-2 in each group were calculated using Image J software (NIH) and are listed in Table II.

Effect of TGF- β 1 on mitochondrial ROS generation in BMSCs. Mitochondrial ROS are strong inducers of cell apoptosis (24). It has been indicated that TGF- β 1 can induce mitochondrial ROS production in cell lineages other than BMSCs (19,25). Flow cytometric data showed that treatment with TGF- β 1 (10 and 20 ng/ml) also markedly increased mitochondrial ROS levels in BMSCs (Fig. 4).

Discussion

BMSCs are the most promising seed cells in stem cell-based transplantation and have been widely used in the clinical treatment of certain diseases, such as neurodegeneration and myocardial infarction (26,27). Generally, BMSCs must be induced *in vitro* for directional differentiation prior to transplantation. TGF- β 1 is one of the most commonly used reagents to induce the directional differentiation of BMSCs into cardiomyocyte-like cells (6-8). In the present study, it was found that the viability of the BMSCs was markedly decreased and the apoptosis rates of the BMSCs were increased following exposure to 10 and 20 ng/ml TGF- β 1 for 24 h. Furthermore, the mitochondrial ROS levels were notably increased in the TGF- β 1-treated BMSCs. These results suggest that TGF- β 1 can induce BMSC apoptosis, with a mechanism that may involve mitochondrial ROS production.

The TGF- β proteins comprise a large family of cell regulatory proteins that plays crucial roles in a series of physiological processes, including cell proliferation, differentiation, apoptosis and autophagy, in numerous cell types (9-11). TGF- β has three main isoforms: TGF- β 1, TGF- β 2 and TGF- β 3 (28). TGF- β 1 was the first-identified isoform in the TGF- β family, and is the most important isoform in the regulation of cell growth and differentiation (29). A number of studies have used TGF- β 1 to induce the cardiomyogenic differentiation of BMSCs and have successfully acquired cardiomyocyte-like cells using this method (6-8); however, several lines of evidence suggest that TGF- β 1 acts to trigger apoptosis of numerous cell types (15-17). In the present study, it was found that TGF- β 1 inhibited the proliferation and promoted the apoptosis of BMSCs.

Bcl-2 and Bax are two important members of the Bcl-2 family, and their ratio (Bax:Bcl-2) determines the level of cell apoptosis (23,24). In the present study it was found that treatment with TGF- β 1 (10 and 20 ng/ml) significantly upregulated Bax but downregulated Bcl-2 protein expression in BMSCs. Annexin V is another apoptotic promoter that preferentially binds phosphatidylserine and promotes cell apoptosis (30). The present results demonstrated that Annexin V was markedly upregulated in BMSCs following exposure to 10 and 20 ng/ml TGF- β 1 for 24 h.

Mitochondrial ROS can induce apoptosis via regulation of the balance between Bcl-2 and Bax (24). It has also been demonstrated that mitochondrial ROS mediate the mitochondrial-dependent cell apoptosis induced by TGF- β in certain non-BMSC lineages, such as fetal hepatocytes (31). Our previous study demonstrated that TGF- β 1 induced mitochondrial ROS generation in BMSCs (20). Consistent with these previous data, the results in the present study also demonstrated that TGF- β 1, at doses of 10 and 20 ng/ml, promoted mitochondrial ROS production in BMSCs. The

TGF- β 1-mediated mitochondrial ROS production may be responsible for its actions on BMSC apoptosis.

In conclusion, the present study has demonstrated that TGF- β 1 can promote the apoptosis of BMSCs. The effect of TGF- β 1 on BMSCs may involve mitochondrial ROS generation.

Acknowledgements

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