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Full-length article

Angiopoietin-1 protects mesenchymal stem cells against serum deprivation and hypoxia-induced apoptosis through the PI3K/Akt pathway[1]

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Key words

mesenchymal stem cells; angiopoietin-1; Tie-2 receptor; apoptosis

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Abstract

Aim: The angiopoietin-1 (Ang1)/Tie-2 signaling system not only plays a pivotal role in vessel growth, remodeling, and maturation, but also reduces apoptosis of endothelial cells, neurons, and cardiomyocytes. However, relatively little is known as to whether Ang1 has a protective effect on mesenchymal stem cells (MSC). The aim of the present study was to investigate the protective effect of Ang1/Tie-2 signaling on MSC against serum deprivation and hypoxia-induced apoptosis, and to determine the possible mechanisms. **Methods:** Hoechst 33342 and terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling staining were used to assess the apoptosis of MSC. The expression of Tie-2, Akt, Bcl-2, Bax, and cleaved caspase-9 and -3 was detected by Western blot analysis. **Results:** This study showed that MSC expressed Tie-2 receptor, and Ang1 induced Tie-2 receptor phosphorylation. The protective effect of Ang1 on MSC was dose-dependent and peaked at 50 μg/L; however, the soluble Tie-2/Fc fusion protein, which acts as an inhibitor by sequestering Ang1, abrogated the anti-apoptotic effect. Ang1 induced Akt phosphorylation, increased the Bcl-2/Bax ratio, and decreased the activation of caspase-9 and -3. All these effects were attenuated by Tie-2/Fc and a phosphatidylinositol 3 kinase (PI3K) inhibitor, wortmannin. **Conclusion:** These results demonstrate that Ang1 can protect MSC against serum deprivation and hypoxia-induced apoptosis; Ang1/Tie-2 signaling and its downstream PI3K/Akt messenger pathway are crucial in the processes leading to MSC survival.

Introduction

Mesenchymal stem cells (MSC) possess multilineage transdifferentiation potential[1] as well as continual self-renewal ability. MSC have become highlight in cell therapy and tissue engineering. After transplantation into the myocardial infarction model *in vivo*, MSC are able to differentiate into endothelial cells, vascular smooth muscle cells, and cardiac-like myocytes[2]. Both animal and clinical studies have demonstrated that MSC are capable of ameliorating cardiac function after myocardial infarction[3-7]. However, MSC have failed to produce satisfactory therapeutic effects in some investigations[8,9], possibly due to poor cell viability after transplantation *in vivo*[10,11]. Therefore, a new method that can improve the survival of MSC needs to be developed.

Angiopoietin-1 (Ang1) was isolated as a ligand of Tie-2 receptor[12], which was first identified in endothelial cells[13,14]. A variety of studies have demonstrated that the Ang1/Tie-2 signaling system plays an important role in vessel growth, remodeling, maturation, and normal lymphangiogenesis[15]. Furthermore, it is a strong anti-apoptotic factor in endothelial cells[16,17], neurons[18], synovial cells[19], and cardiomyocytes[20]. It is not known whether Ang1 also has a similar anti-apoptotic effect on MSC to improve cell survival after transplantation. In this study, experiments were designed to determine the effect of Ang1 on MSC viability under serum deprivation (SD) and hypoxic conditions *in vitro*, and the possible mechanisms behind it.

Materials and methods

Recombinant human Ang1, Tie-2 receptor–Fc (Tie-2/Fc)

fusion protein, monoclonal Tie-2 antibody, and the antiphospho-Tie-2 (Y992) antibody were obtained from R&D Systems (Minneapolis, MN, USA). Akt, phospho-Akt, Bcl-2, Bax, and cleaved caspase-9 and -3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The *In situ* cell death detection kit, POD (Roche, Mannheim, Germany), wortmannin (Alexis Biochemicals, Lausen, Switzerland), Hoechst 33342, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) were also used in the study.

MSC isolation and expansion The MSC were isolated and harvested as previously described[21]. In brief, Sprague–Dawley rats were sacrificed in accordance with the methods approved by the Animal Care and Utilization Committee of Zhejiang University (Hangzhou, China), and bone marrow samples were collected from the femoral and tibial cavities with basal Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA). Subsequently, nucleated cells were separated with 1.073 g/mL Percoll solution (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and seeded into 50-cm^2 flasks with DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cultures were maintained at 37 °C in a 5% CO_2 incubator. After 24 h, non-adherent cells were removed, and adherent cells were washed 3 times with phosphate-buffered saline (PBS). The culture medium was changed every 3–4 d, and the cells were subcultured when confluency approached approximately 80%. The spindle-shaped, adherent MSC were expanded and cultured no more than 5 passages before use.

The cells were determined by fluorescence-activating cell sorting (Beckman Coulter, Fullerton, CA, USA) analysis before the experiments, using directly conjugated antibodies against CD44 [fluorescein–isothiocyanate conjugated (FITC); Caltag, Carlsbad, CA, USA], CD45 (FITC, Caltag, USA), and CD90 (phycoerythrin-conjugated; Caltag, USA)[21].

SD/hypoxia and Ang1 treatment protocol A well-established MSC apoptotic model induced by SD and hypoxia *in vitro* was followed in this study[22]. Briefly, the MSC culture medium was removed and the cells were washed twice with PBS before being plated in serum-free DMEM containing the required treatment. Flasks were kept in a modular incubator chamber (Billups–Rothenberg, Del Mar, CA, USA) infused with mixed gas (95% N_2 and 5% CO_2) until the concentration of oxygen was lower than 0.5% at 37 °C.

Ang1 was reconstituted according to the manufacturer's protocol. Concentrations of 12.5, 25, 50, 100, and 200 μg/L were selected for this study. The concentration of 50 μg/L was used to detect the protein expression after confirmation of its maximum effect on MSC apoptosis. The MSC were exposed to Ang1 for 24 h before the assessment of apoptosis and protein expression. To detect Akt phosphorylation, MSC were incubated in SD and a hypoxic environment for 24 h before being exposed to 50 μg/L Ang1 for 25 min.

Measurement of apoptosis

Morphological changes Hoechst33342 was used for the evaluation of chromosomal condensation and nuclear fragmentation. The cells were carefully washed with PBS and fixed in freshly prepared 4% paraformaldehyde for 30 min. The samples were exposed to 5 μg/mL Hoechst 33342 for 10 min at room temperature under dark conditions. Subsequently, all of the samples were observed under a phase contrast microscope and a fluorescence microscope. Apoptotic cells were characterized by morphological changes, such as cell shrinkage, nuclear condensation, and fragmentation.

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling MSC apoptosis was also determined by terminal deoxynucleotidyl transferase-mediated-dUTP nick-end labeling (TUNEL) assays. TUNEL staining was performed mainly according to the manufacturer's protocol with some modifications. Briefly, the MSC were carefully washed twice with PBS and fixed with freshly prepared 4% paraformaldehyde for 1 h at room temperature, followed by incubation with a blocking solution (3% H_2O_2 in methanol) for 30 min. The MSC were permeabilized with a freshly prepared solution containing 0.5% Triton X-100 in 0.1% sodium citrate for 1 h at 37 °C. The TUNEL reaction mixture (50 μL) was then added to the samples and incubation continued for another 1 h at 37 °C in a humidified atmosphere under dark conditions. Later, 50 μL of converter POD was added to the samples, followed by 30 min incubation at 37 °C. The chromogenic reaction was carried out with 3,3'-diaminobenzidine and terminated by the addition of tap water. Finally, the MSC were counterstained with hematoxylin and analyzed by a light microscope.

Cell survival assessment The MTT assay was used to evaluate cell viability. The MSC were cultured in 96-well tissue culture plates, and 1×10^4 cells were equally seeded into each well. MTT was added at a final concentration of 0.5 mg/mL after different treatments. Four hours later, the medium was removed and DMSO was applied to solubilize the purple formazan. The results were read with an ELISA reader at 570 nm.

Western blot analysis After the MSC were treated in different conditions for the required times, the cells were scraped from the flasks and harvested in 1.5 mL Eppendorf tubes. RIPA cell lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, and 1% NP-40 (1 mmol/L phenylmethylsulfonyl fluoride and 10 mmol/L

NaF were added before use) was applied to lyse the MSC. The tubes were vortexed until the cells were lysed completely, followed by centrifugation at 12 000×*g* for 30 min. The protein concentrations of the supernatant were determined by the Bio-Rad DC protein assay method (Bio-Rad, Hercules, CA, USA). All procedures were performed at 4 °C.

The proteins (30-100 μg) were then loaded onto SDS–polyacrylamide gels, separated according to the molecular weight, and subsequently, electrophoretically transferred onto polyvinylidene difluoride membranes using a Bio-Rad wet transfer system. After blockage with 0.1% Tween in Tris-buffered saline containing 5% milk at room temperature for 1 h, the membranes were incubated with specific primary antibodies by gentle agitation overnight at 4 °C. The conjugation of the primary antibodies with horseradish peroxidase-marked secondary antibodies was kept at room temperature for 1 h. Finally, bands were obtained by using an enhanced chemiluminescence detection system (Biological Industries, Beit Haemek, Israel) and were analyzed by Image-pro plus version 5.02 (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis Data were expressed as mean±standard deviation and were analyzed with the commercially available statistical software package, SPSS 13.0 (SPSS, Chicago, IL, USA). One-way ANOVA test was performed, and *post-hoc* multiple comparisons were done with S-N-K. Statistical significance was defined as $P<0.05$.

Results

Ang1 protects MSC against apoptosis and cell death induced by SD and hypoxia Hoechst and TUNEL staining were used to evaluate the protective effect of Ang1 on MSC against SD and hypoxia-induced apoptosis. After 24 h of treatment, cell shrinkage and nuclear condensation were remarkably increased in the SD/hypoxia group. Surprisingly, Ang1 clearly exerted a protective effect on MSC, as demonstrated by morphological and nuclear changes (Figure 1A, 1B). Ang1 reduced the apoptotic rate in a dose-dependent manner and peaked at the concentration of 50 μg/L (Figure 1C,2A,2B). However, the soluble Tie-2/Fc fusion protein, which acts as an inhibitor by sequestering Ang1 to block its

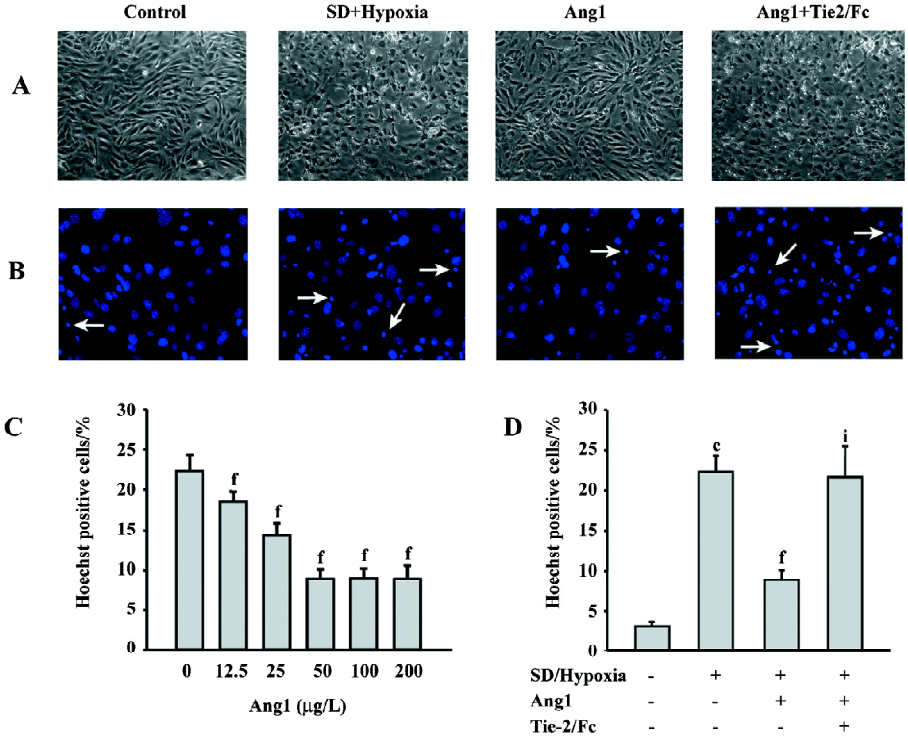

Figure 1. Effect of Ang1 on apoptosis induced by SD and hypoxia in MSC. MSC were cultured in 4 groups: 10% FBS and normoxia (control group), SD and hypoxia (the concentration of O_2 <0.5%), SD+hypoxia+Ang1 (Ang1 group), and SD+hypoxia+Ang1+Tie-2/Fc (Ang1+Tie-2/Fc group). All 4 groups were cultured for 24 h followed by apoptosis evaluation. (A) morphological alteration was detected by a phase contrast microscope (×40). (B) Hoechst 33342 staining. White arrows show the fragmented and condensed nuclei of apoptotic cells. Magnification of fluorescent microscope was ×200. (C) effect of Ang1 on MSC apoptosis. (D) 10-fold of Tie-2/Fc (500 μg/L) completely blocked the protective effect of Ang1. $n=3$. $^{c}P<0.01$ *vs* control group; $^{f}P<0.01$ *vs* SD+hypoxia group, $^{i}P<0.01$ *vs* Ang1 group.

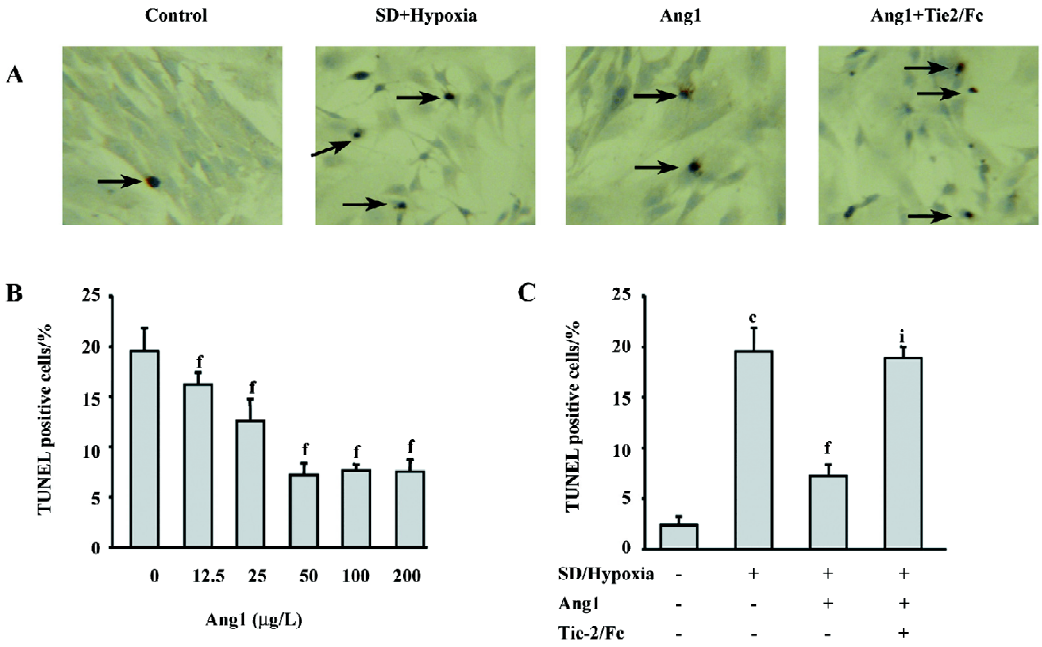

Figure 2. Effect of Ang1 on MSC apoptosis determined by TUNEL staining. (A) brown nucleus staining (black arrows) indicated apoptotic MSC. Magnification ×200. (B) Ang1 showed dose-dependent protection on MSC. (C) 10-fold of Tie-2/Fc (500 μg/L) completely blocked the protective effect of Ang1. n=3. ^{c}P<0.01 *vs* control group; ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

function, abrogated its protective effect (Figure 1D,2C). Similarly, Ang1 increased MSC viability of during SD and hypoxia while Tie-2/Fc inhibited a positive effect (Figure 3).

Ang1 induces Tie-2 receptor phosphorylation Western analysis demonstrated that the Tie-2 protein expression in MSC was slightly upregulated after SD and hypoxia insults, while the Ang1 application did not affect its level (Figure 4A). However, in the presence of Ang1, the phospho-Tie-2 expression of MSC was apparently increased, and the ratio of p-Tie-2/Tie-2 was critically increased in the Ang1 group as compared with the SD and hypoxia group (P<0.001). The ratio was strikingly decreased in the Ang1+Tie-2/Fc group (Figure 4B).

Ang1 activates Akt phosphorylation To evaluate downstream Ang1/Tie-2 signaling, the protein expressions in the phosphatidylinositol 3 kinase (PI3K)/Akt messenger pathway were examined by Western blot analysis. The data suggested that SD and hypoxia critically inhibited the phosphorylation of Akt (Ser473) in comparison with the control group, while exposure to Ang1 (50 μg/L) resulted in an increase in Akt phosphorylation. The ratio of p-Akt/Akt was obviously increased in the Ang1 group compared with the SD and hypoxia group. The presence of wortmannin (a selective inhibitor of PI3K) attenuated Akt phosphorylation induced by Ang1 (Figure 5).

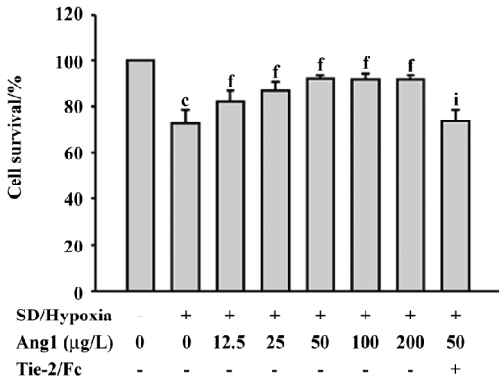

Figure 3. Effect of Ang1 on MSC viability. Cell viability was remarkably reduced in the SD and hypoxia group. Ang1 increased cell viability in a dose-dependent manner; however, the effect was abrogated by Tie-2/Fc. Viability of the control group was arbitrarily presented as 100%; the viability of the other groups was calculated by (optical density [*OD*] value of the treated group–blank)/(*OD* value of control group–blank)×100%. n=3. ^{c}P<0.01 *vs* control group; ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

Ang1 increases the Bcl-2/Bax ratio Furthermore, we investigated the Bcl-2 family proteins to confirm the effect of SD and hypoxia and Ang1 on apoptosis-related proteins. Western blot analysis was performed and showed that SD and hypoxia decreased the ratio of Bcl-2/Bax, an important

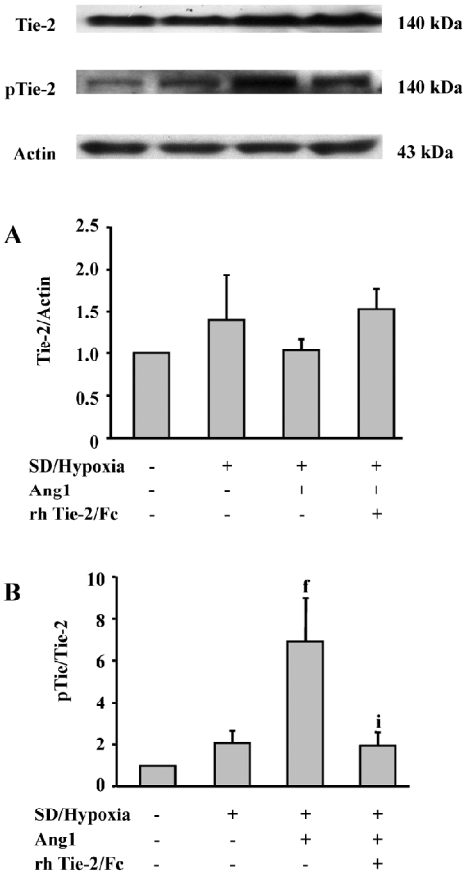

Figure 4. Expression of Tie-2 in MSC and the activation of Tie-2 by Ang1. (A) no obvious difference in Tie-2 expression was observed in the 4 groups. (B) Ang1 (50 μg/L) remarkably increased the active Tie-2 expression, whereas Tie-2/Fc (500 μg/L) attenuated the effect. Densitometric analysis was used to calculate the relative ratio of Tie-2/actin and phospho-Tie-2/total Tie-2. Ratio for the control group was arbitrarily presented as 1. n=3. ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

factor determining the susceptibility of apoptosis. The Bcl-2/Bax ratio increased by approximately 4-fold in response to Ang1 administration, whereas this effect was blocked by the inhibition of Ang1 and the PI3K/Akt pathway (Figure 6).

Ang1 decreases caspase-9 and -3 activation The results also illustrated the influence of Ang1 on SD and hypoxia-induced caspase activation. Almost no cleaved caspase-9 and -3 expressions were detected in the control group, while after SD and hypoxia treatment, both cleaved caspases were remarkably elicited. The intensities of these bands were attenuated when Ang1 was present in the culture medium. However, wortmannin, a specific PI3K inhibitor, and Tie-2/Fc reversed the inhibitory effect of Ang1 (Figure 7).

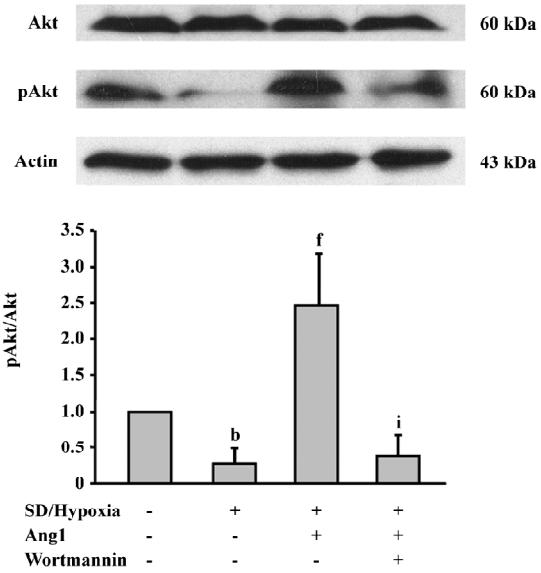

Figure 5. Activation of Akt by Ang1 treatment. MSC were cultured in SD and hypoxia for 24 h before exposed to the Ang1 (50 μg/L) for 25 min. In the fourth lane, wortmannin (100 nmol/L) was added to the cells 1 h before exposure to Ang1 (50 μg/L). Densitometric analysis was used to calculate the relative ratio of phospho-Akt/total Akt. Ratio for the control group was arbitrarily presented as 1. n=3. ^{b}P<0.05 *vs* control group; ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

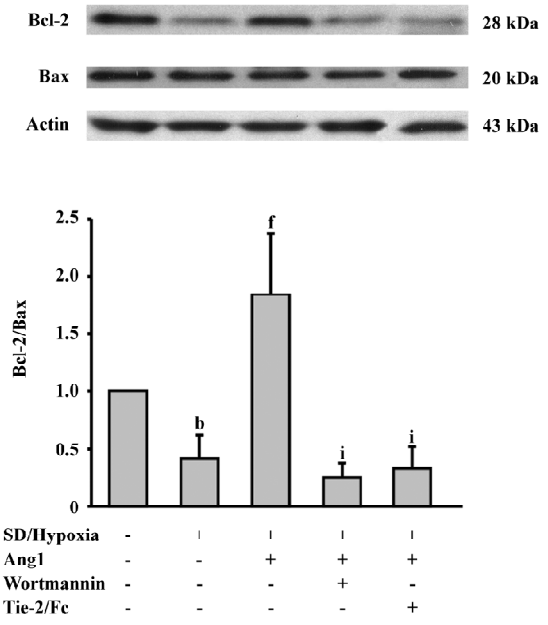

Figure 6. Effect of Ang1 on Bcl-2 and Bax expression. MSC were treated in the presence or absence of Ang1 (50 μg/L) and wortmannin (100 nmol/L) for 24 h. Densitometric analysis was used to calculate the relative ratio of Bcl-2/Bax. Ratio for the control group was arbitrarily presented as 1. n=3. ^{b}P<0.05 *vs* control group; ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

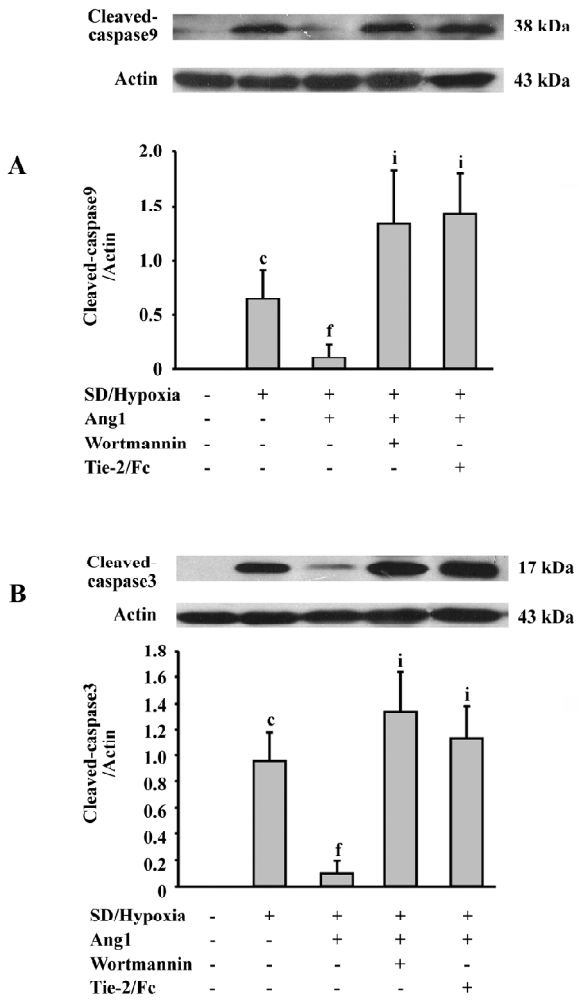

Figure 7. Effect of Ang1 on caspase-9 and -3 activation. MSC were treated in the presence or absence of Ang1 (50 μg/L) and wortmannin (100 nmol/L) for 24 h. Densitometric analysis was applied to calculate the relative ratio of cleaved caspase-9/actin (A) and cleaved caspase-3/actin (B). Ratio for the control group was arbitrarily presented as 1. n=3. ^{c}P<0.01 *vs* control group; ^{f}P<0.01 *vs* SD+hypoxia group; ^{i}P<0.01 *vs* Ang1 group.

Discussion

In this study, we explored a new strategy to improve the survival of MSC *in vitro* and showed favorable results for the first time that Ang1 could improve MSC viability after SD and hypoxia-induced apoptosis. After exposure to Ang1, the Tie-2 protein became remarkably phosphorylated in MSC. Ang1 also induced the phosphorylation of Akt, increased the Bcl-2/Bax ratio, and decreased cleaved caspase-9 and -3 activation. Interestingly, all these effects were abrogated by Tie-2/Fc and wortmannin. Therefore, Ang1/Tie-2 and its downstream PI3K/Akt signal messenger pathway were involved in the mechanism of the Ang1 protection of MSC from SD and hypoxia-induced apoptosis.

Recent stem cell research in cardiology suggested that MSC are promising in regenerating the infracted myocardium[23]. However, most transplanted cells die within 4 d after transplantation to the ischemic heart [24]. Donor cell survival after transplantation remains among the major limitations that greatly influence the outcome of heart cell therapy. With emerging interest in gene modification, Ang1, Akt, and the HO-1 gene have been used to modify MSC. The engraftment of such modified MSC further improved cell survival and cardiac functions[24-26] . However, the safety of gene therapy has become an increasing public concern, and a variety of problems still remain[27,28]. In the present study, a much safer approach in the protein level and with potential clinical value was investigated.

The data used in the present study demonstrated the protective effect of Ang1 on MSC and showed the role of PI3K/Akt in Ang1/Tie-2 signaling, which was consistent with the observations on endothelial cells and neurons[18,29]. Akt has been shown to become phosphorylated to regulate the function of many cellular proteins involved in metabolism, survival/apoptosis, differentiation, and proliferation[30]. Increased Akt activity in genetically-modified MSC has been shown to protect cells against SD and hypoxia-induced apoptosis[26]. Consistent with previous reports, Ang1 promoted Akt phosphorylation and reduced MSC apoptosis in our study.

There are two major signaling pathways of apoptosis: the death receptor pathway and the mitochondrial pathway[31,32]. A recent study showed that SD and the hypoxia-induced apoptosis of MSC may not be affected by the death receptor pathway[22]. Therefore, Bcl-2, Bax, and caspase-9 and -3 levels were examined in this study to confirm the mitochondrial pathway, which may be involved in the regulation of apoptosis. The presence of an anti-apoptotic molecule, such as Bcl-2, can inhibit the activation of Bax following a death signal[33]. The ratio of Bcl-2/Bax is an important indicator of cell susceptibility to apoptosis[34,35]. Caspase-9 is activated by apoptosome, which is composed of cytochrome *c*, Apaf-1, and procaspase-9. It plays a critical role in the mitochondrial apoptosis pathway[32]. Caspase-3 has been demonstrated as a key mediator of mitochondrial events of apoptosis[36] and can be activated by SD and hypoxia in MSC[22]. In accordance with previous studies, the reduction of MSC apoptosis of by Ang1 administration correlated with an increase of the Bcl-2/Bax ratio and a decrease of caspase-9 and -3 activation in this study.

In conclusion, data in this study demonstrated that Ang1 protects MSC against SD and hypoxia-induced apoptosis.

The results indicate that Ang1/Tie-2 signaling and its downstream PI3K/Akt messenger pathway are crucial for MSC survival, providing a promising approach for improving MSC viability.

Author contribution

Jian-an WANG designed research and provided financial support; Xian-bao LIU performed research; Jun JIANG wrote paper; Chun GUI and Xin-yang HU analyzed data; Mei-xiang XIANG contributed hypoxic chamber.

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