TGF-β2 treatment enhances cytoprotective factors released from embryonic stem cells and inhibits apoptosis in infarcted myocardium

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Singla DK, Singla RD, Lamm S, Glass C. TGF-β2 treatment enhances cytoprotective factors released from embryonic stem cells and inhibits apoptosis in infarcted myocardium. Am J Physiol Heart Circ Physiol 300: H1442–H1450, 2011. First published February 4, 2011; doi:10.1152/ajpheart.00917.2010.—We investigated whether factors released from mouse embryonic stem (ES) cells primed with and without transforming growth factor (TGF)-β2 inhibit iodoacetic acid (IAA)- and H2O2-induced apoptosis in the cell culture system as well as after transplantation in the infarcted heart. We generated conditioned media (CMs) from ES cells primed with and without TGF-β2 and determined their effects on IAA- and H2O2-induced apoptosis in H9c2 cells. We also transplanted both ES-CMs in the infarcted heart to determine the effects on apoptosis and cardiac function after myocardial infarction (MI) at day (D)1 and D14. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, apoptotic ELISA, and cell viability data demonstrated significantly (P < 0.05) reduced apoptosis with ES-CM compared with controls in both cell culture models. Moreover, TGF-β2-primed ES-CM (T-ES-CM) demonstrated enhanced beneficial effects, with further reduced (P < 0.05) apoptosis compared with ES-CM, suggesting the presence of additional cytoprotective released factors after TGF-β2 treatment. Next, our in vivo apoptosis data suggested significant decrease in apoptosis in both ES-CMs compared with MI alone at D1 and D14. Notably, T-ES-CM demonstrated significant (P < 0.05) inhibition of apoptosis and fibrosis with improved cardiac function compared with ES-CM at D14, whereas no such effects were observed at D1. Next, we confirmed that apoptosis is mediated through a prosurvival Akt pathway. Moreover, we determined that after TGF-β2 treatment there was a two- to fivefold increase in cytoprotective released factors (interleukin-10, stem cell factor, tissue inhibitor of matrix metalloproteinase-1, and VEGF) with T-ES-CM compared with ES-CM. In conclusion, we suggest that factors released from ES cells with and without TGF-β2 treatment contain antiapoptotic factors that inhibit apoptosis in vitro and in vivo. We also suggest that T-ES-CM demonstrates additional beneficial effects that provide useful information for future therapeutic applications in regenerative medicine.

APOTOPSIS IS CONSIDERED a structurally and biochemically distinct form of cell death and is required for normal organ development, deletion of harmful nonfunctional cells, and control of cell number (2, 28). Significant increase in cell death due to apoptosis or necrosis plays a major role in the development of various diseases, including autoimmune, neurodegenerative, and cardiovascular disorders (2, 10, 11, 28, 33). Gottlieb et al. (16) first reported the presence of apoptosis during myocardial ischemia-reperfusion. Following this first report, there have been a number of reports that confirm the association of apoptosis and necrosis in the development and progression of heart diseases including myocardial infarction (MI) (2, 28). Major heart cell types such as cardiomyocytes, vascular smooth muscle cells, and endothelial cells have been shown to die as a result of both necrosis and apoptosis (2, 28). Cell death may be partially offset by differentiation of endogenous cardiac cells into cardiomyocytes in the adult heart (4, 5). Moreover, cell transplantation to treat heart disease has been studied in numerous small- and large-animal models (4, 5, 17, 18). These transplanted stem cells include c-kit+, sca-1, CD34+, skeletal myoblasts, bone narrow stem cells, fetal and embryonic cardiomyocytes, and mouse embryonic stem (ES) cells (4, 5, 17, 18, 26).

Recent data suggest that mouse ES cells transplanted in the heart can differentiate into cardiomyocytes, vascular smooth muscle, and endothelial cells after MI (36). However, transplanted stem cell data demonstrate significant low levels of engraftment and cardiac myocyte differentiation compared with improved cardiac function. A recent study suggests that, when primed with transforming growth factor (TGF)-β1, ES cells exhibit increased engraftment and differentiation after transplantation (21). We have previously shown (40) that procardiac growth factor TGF-β2-primed ES cells enhance cardiomyocyte differentiation in the cell culture system. We also demonstrated in recent studies (38) that factors released from ES cells are antiapoptotic and inhibit H2O2-induced apoptosis in H9c2 cardiomyoblast cells. However, whether substances released from ES cells primed with growth factors will enhance inhibition of apoptosis remains unknown.

Accordingly, we hypothesized that TGF-β2-primed ES cells will release increased amounts of cytoprotective factors, which will enhance the inhibition of apoptosis in the various models of apoptosis in vitro and in vivo. To test this hypothesis, we prepared conditioned media (CMs) from TGF-β2-primed or unprimed ES cells and induced apoptosis in the cell culture model with iodoacetic acid (IAA) and H2O2, simulating oxidative stress typical of the post-MI heart. Moreover, we transplanted factors released from TGF-β2-primed ES cells in the infarcted heart to understand their effects on apoptosis and cardiac function. We present data for the first time showing that cytoprotective factors released from ES cells primed with the procardiac growth factor TGF-β2 enhance inhibition of apoptosis in vitro and in vivo.
Preparation of TGF-β2-primed ES cell conditioned medium. CGR8 mouse ES cells were passaged and maintained as we reported previously (38, 39). In brief, ES cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing nutrient leukemia inhibitory factor (LIF) and other growth factors including sodium pyruvate, glutamine, β-mercaptoethanol, penicillin-streptomycin, nonessential amino acids, and 15% ES cell-qualified fetal bovine serum (Invitrogen). Rat cardiomyoblast cell line H9c2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM according to instructions provided by the ATCC and reported previously (38, 39).

Mouse ES cells (1.6 million) were placed on 150-mm² gelatinized petri dishes containing cell culture medium with LIF for 24 h. Cells were then treated with or without TGF-β2 (8 ng/ml) in fresh cell culture medium without LIF. After 48 h, supernatants were collected, filtered with a 0.2-μm filter, and labeled as TGF-β2-ES-CM (T-ES-CM) or without TGF-β2 as ES-CM for further use to examine their effects on apoptosis in vitro and in vivo studies.

Preparation of apoptotic cell culture model. H9c2 cells were cultured (800 cells/cm²) for 24 h. The optimal dose of IAA (Sigma) was assessed by exposing the cells to varying concentrations of IAA ranging from 50 to 400 μM for 1 h. The cells were then treated with fresh cell culture medium and cultured for an additional 24 h. On the basis of cellular morphology, we observed that cells treated with 100 μM IAA demonstrated the greatest amount of cell death. Therefore, in subsequent experiments, H9c2 cells were exposed to 100 μM IAA for 1 h, followed by fresh cell culture medium, ES-CM, or T-ES-CM. For H2O2-induced apoptotic cell death, a concentration of 400 μM was used, as we reported previously (38, 39). The number of surviving cells was examined by Trypan blue staining.

Apoptosis was examined by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining and an apoptotic ELISA kit.

Apoptosis. As previously reported, we used TUNEL staining for apoptosis detection (38). In brief, H9c2 cells were exposed to IAA (100 μM) for 1 h and H2O2 for 2 h, followed by fresh cell culture medium, ES-CM, or T-ES-CM. Cells were cultured for an additional 24 h, washed with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde in PBS. Cell permeabilization was performed with 0.1% Triton X-100 in 0.1% sodium citrate followed by proteinase K (25 μg/ml in 100 mM Tris-HCl), and an in situ apoptotic cell death detection kit (TMR red; Roche Applied Science) was used to detect apoptotic cells as described in the manufacturer’s protocol. Apoptotic cells were also colabeled with caspase-3 and cardiac α-actin antibodies to determine the presence of caspase-3 in apoptotic cardiac myocytes. For caspase-3 staining, sections were incubated with active anti-caspase-3 rabbit polyclonal (1:1,000 dilution; Cell Signaling) and sarcomeric cardiac α-actin mouse monoclonal (1:30; Sigma) antibodies. Sections were incubated in a humidified chamber at 37°C for 1 h, washed with PBS, and incubated with secondary antibodies: anti-rabbit Alexa 660 (Invitrogen) or anti-mouse Alexa 488 (MOM kit, Vector Laboratories). Sections were washed and mounted with Vectashield medium containing DAPI (Vector Laboratories) to visualize all nuclei, which were stained blue. Apoptosis and caspase-3-positive cells were identified with an Olympus fluorescence microscope and a LEICA laser scanning confocal microscope. Apoptotic nuclei were quantified in the infarct and peri-infarct areas of the heart in one or two heart sections from five to eight different hearts as reported by us (37) and others (23, 30, 42) previously. The percentage of apoptotic nuclei per section was determined by the manually counted total number of red TUNEL-stained nuclei divided by the total number of blue nuclei stained with DAPI multiplied by 100 at ×20 magnification.

Akt activity assay. The Phospho-AKT1 ELISA kit (Exalpha Biological) is specifically designed to measure the active form of Akt1(S473), which was used in the present study. In brief, hearts were homogenized in radioimmunoprecipitation assay (RIPA) buffer and protein concentration was estimated as previously described (40). The manufacturer’s instructions were strictly followed, and the reaction developed appeared yellow in color. The color intensity was quantified in a microtiter plate reader (Bio-Rad) at 450 nm.

Masson’s trichrome staining. To determine fibrosis, heart sections were prepared and stained with Masson’s trichrome staining as we published previously (37). Trichrome-stained areas appeared blue in color if there was fibrosis present in the heart. We examined the infarct, peri-infarct, and noninfarcted regions of the heart from all groups, and fibrosis was quantified by measuring the total blue area per square millimeter with the NIH ImageJ program.
Determination of factors released in ES-CMs. T-ES-CM, ES-CM, and control cell culture medium were generated and submitted to Rules-Based Medicine (Austin, TX), where cytokine and growth factor analysis was performed (with Luminex technology) as we reported previously (38). In brief, a sample is introduced into microspheres of the rodent antigen mitogen-activated protein, thoroughly mixed, and incubated at room temperature for 1 h. Multiplexed cocktails of biotinylated and reporter antibodies for each multiplex are then added and incubated for an additional 1 h at room temperature. Next, multiplexes are incubated with streptavidin-phycoerythrin solution, and the developed reaction is analyzed in a Luminex 100 instrument. Data were analyzed with proprietary data analysis software. This rodent antigen-specific kit detected a total of 59 cytokines and growth factors present in the CMs.

Data analysis. Statistical significance of differences between values was assessed when \( P < 0.05 \) by Student's \( t \)-test or one-way ANOVA. Data are expressed and plotted as means ± SE.

Fig. 1. Effects of iodoacetic acid (IAA) treatment on H9c2 cell viability and apoptosis. A, left: representative photomicrographs of control H9c2 cells and H9c2 cells exposed to IAA, IAA + embryonic stem (ES) cell conditioned medium (CM) (ES-CM), and IAA + transforming growth factor (TGF)-β2-primed ES-CM (T-ES-CM) (×20). Right: no. of H9c2 cells viable after exposure to 50–400 μM concentrations of IAA. B, left: quantitation of apoptotic nuclei (n = 5–7 fields/well in each condition). Data are from a set of 4 or 5 independent experiments. Middle: quantitation by apoptotic ELISA (see MATERIALS AND METHODS). Data are from a set of 6–8 independent experiments. Right: quantitation of percentage cell viability examined by Trypan blue staining criteria (see MATERIALS AND METHODS). Data are from a set of 4–6 independent experiments. *\( P < 0.05 \) vs. IAA group; #\( P < 0.05 \) vs. IAA and ES-CM groups.
RESULTS

To investigate the effect of IAA-induced apoptosis in H9c2 cells, cells were treated with various concentrations (50–400 μM) of IAA as shown in Fig. 1. H9c2 cells treated with IAA demonstrated morphological changes and significant (P < 0.05) decrease in cell survival (Fig. 1A). We determined that the 100 μM concentration of IAA was optimal, as a significant decrease in cell survival (~50%) was observed.

IAA-induced apoptosis in H9c2 cells was first detected by TUNEL staining. In Fig. 1B, blue nuclei stained with DAPI indicate the total number of nuclei (Fig. 1B, a–d), whereas red fluorescence shows TUNEL-stained nuclei (Fig. 1B, e–h). Merged images of nuclei are demonstrated in Fig. 1B, i–l. We determined a significantly increased percentage of TUNEL-positive nuclei with IAA treatment, and this increase was inhibited with both ES-CM treatments (Fig. 1C, P < 0.05). Moreover, T-ES-CM showed enhanced decrease in apoptosis compared with untreated ES-CM (Fig. 1C). Furthermore, we determined quantitative apoptosis measured by an ELISA assay. In Fig. 1C, data show significantly (P < 0.05) reduced apoptosis with both ES-CMs compared with cell culture medium. Next, cell viability was determined with Trypan blue staining and cell morphology criteria. We demonstrated significant decrease in cell survival following treatment with IAA, and this decrease was inhibited with both ES-CMs (Fig. 1C, P < 0.05). Furthermore, T-ES-CM showed significant increase in cell survival compared with ES-CM.

Next, we examined the effects of TGF-β2-released factors from ES cells on H2O2-induced cell death. Effects of T-ES-CM compared with ES-CM demonstrated significantly (P < 0.05) decreased H2O2-induced apoptosis in H9c2 cells. Apoptosis was confirmed by TUNEL staining (Fig. 2A) and apoptotic ELISA (Fig. 2B). Furthermore, T-ES-CM significantly (P < 0.05) enhanced cell viability as determined by the Trypan blue method (Fig. 2C). These data suggest that T-ES-CM contains increased concentrations of cytoprotective factors that inhibit apoptosis.

Thereafter, we wanted to confirm our findings that both ES-CMs are antiapoptotic in vivo. We generated MI in mice, and both ES-CMs were transplanted into the infarcted heart to examine their effects on apoptosis after MI at D1 and D14. Apoptosis was confirmed with TUNEL and caspase-3 immunostaining. Figure 3, A and B, shows significantly (P < 0.05) decreased apoptosis in both MI+ES-CM and MI+T-ES-CM groups compared with the MI group at D1 and D14. Importantly, T-ES-CM further significantly reduced apoptosis at D14 compared with ES-CM. However, no significant difference was observed at D1. Moreover, TUNEL-stained apoptotic nuclei were also positive for cleaved caspase-3, suggesting that caspase-3 mediates apoptosis in the heart (Fig. 3D). Next, we determined whether apoptosis was mediated by Akt, a prosurvival protein. Our data suggest that transplanted CM significantly (P < 0.05) increased levels of phosphorylated Akt1 compared with the MI group (Fig. 4). Moreover, the T-ES-CM group showed further significant (P < 0.05) increase in Akt compared with ES-CM and MI groups at D14 (Fig. 4). These data suggest that inhibition of apoptosis following transplantation of both CMs is mediated through the Akt pathway. Furthermore, we determined the effects of both CMs on cardiac fibrosis. Cardiac fibrosis was significantly inhibited with both CMs after transplantation (Fig. 5). Additionally, inhibition of fibrosis with T-ES-CM was significantly (P < 0.05) greater compared with ES-CM (Fig. 5). Next, fractional shortening and ejection fraction were also determined to understand the effects of transplanted ES-CMs on cardiac function. Our data suggest that both ES-CMs significantly (P < 0.05) improved fractional shortening and ejection fraction compared with the MI group at D14 after MI (Fig. 6). In addition, T-ES-CM also demonstrated further improvement in fractional shortening and ejection fraction compared with ES-CM (Fig. 6). Therefore, our cardiac functional data are in accordance with the apoptosis and fibrosis data observed in the present study.

Whether TGF-β2-treated ES-CM contains additional specific factors compared with untreated ES-CM was determined. The detailed list of 59 cytokines and growth factors examined in the CMs is listed in Supplemental Table S1.1 Cytoprotective cytokine and growth factors released in the CM by TGF-β2-primed ES cells were present at levels greater than those found in the unprimed ES cells and control cell culture medium.

1 Supplemental Material for this article is available online at the Journal website.

Fig. 2. Quantitative analysis of H2O2-induced apoptosis determined by TUNEL staining (A), apoptotic ELISA (B), and Trypan blue staining (C) after exposure to H2O2. A: quantitation of apoptotic nuclei (n = 6–8 fields/well in each condition). Data are from a set of 5–7 independent experiments. B: quantitation by apoptotic ELISA (see MATERIALS AND METHODS). Data are from a set of 6–8 independent experiments. C: quantitation of percentage of cell viability examined by Trypan blue staining (see MATERIALS AND METHODS). Data are from a set of 4–6 independent experiments. *P < 0.05 vs. H2O2 group; #P < 0.05 vs. H2O2 and ES-CM groups.
(containing serum background levels), as shown in Supplemental Table S2. Four major cell-protective cytokines and growth factor proteins were released in the TGF-β2-primed or unprimed ES-CMs, specifically, interleukin-10 (27- to 48-fold), stem cell factor (9- to 20-fold), vascular endothelial growth factor (169- to 1,008-fold), and tissue inhibitor of matrix metalloproteinase (TIMP)-1 (142- to 595-fold), which were significantly different from their levels in cell culture medium (Table 1). Moreover, we suggest that TGF-β2 treatment further enhanced the levels of released cytoprotective factors.

**DISCUSSION**

Two major types of cell death have been reported to date, apoptosis and necrosis (2, 10, 28). Apoptosis is programmed...
cell death controlled by the cellular genetic material (2, 10, 28). In contrast, necrosis is a structurally and biochemically distinct form of naturally occurring cell death (2, 10, 28). Apoptosis and necrosis have been reported to be involved in the progressive deterioration of MI, ischemia-reperfusion, and dilated cardiomyopathy hearts (2, 10, 28). Various stimuli such as TNF-α, doxorubicin, and H2O2 induce apoptosis in cell culture models of isolated cardiomyocytes and cardiomyoblasts (H9c2 cells) (2, 10, 27, 28). However, whether IAA, an alkylating agent that blocks the glycolytic pathway and oxidant stressor (13), can induce apoptosis in the cardiomyoblast cell line H9c2 remains unknown. In the present study, we provide evidence that IAA-treated H9c2 cells show significantly increased cell death. Next, cell death detection methods such as TUNEL staining and apoptotic ELISA confirm apoptosis, suggesting that IAA-induced cell death in the H9c2 cells is apoptotic in nature. Moreover, in the present study apoptosis induced in H9c2 cells by H2O2 and IAA were similar in nature as determined by apoptosis assays. However, further detailed stepwise studies at various time points as well as different concentrations of the apoptotic stimuli (H2O2 and IAA) are needed to delineate whether apoptosis is different with these two stimuli. Our data are consistent with previous findings, which suggests that IAA induces the apoptotic type of cell death in various cell lines such as astrocytes, rat primary neural cultures, and renal epithelial cells (13, 34, 41).

We recently reported (38, 39) that factors released from surviving as well as dying cells after exposure to H2O2 protect against H2O2-induced apoptosis in H9c2 cells. Similarly, it has been reported that factors released from Akt-modified mesenchymal stem cells inhibit stress-induced apoptosis in isolated cardiomyocytes (14). In the present study, we provide evidence that released factors from ES cells significantly inhibit IAA- and H2O2-induced apoptosis in H9c2 cells. These data are consistent with our previous report (38) indicating that released factors from ES cells contain antiapoptotic factors and inhibit H2O2-induced apoptosis.

We previously reported (40) that TGF-β2 enhances beating cardiomyocytes in embryoid bodies derived from ES cells. Moreover, TGF-β2 inhibits apoptosis in different cell types including cerebellar granule cell precursors and osteoblasts (8, 9). Accordingly, we treated ES cells with TGF-β2 and generated ES-CM (see MATERIALS AND METHODS) to test the hypothesis that factors released from TGF-β2-primed ES cells could enhance cytoprotection in IAA- and H2O2-induced apoptotic cell death. Our data suggest that TGF-β2-released factors have additional protective effects compared with untreated ES-CM on IAA- or H2O2-induced cell death in H9c2 cells. The present study offers new opportunities to investigate whether additional protective effects of released factors following treatment with TGF-β2 can also be seen in other organ-specific cell types such as osteoblasts and endothelial and vascular smooth muscle cells. Although we demonstrate that TGF-β2-primed ES cells release cytoprotective factors in the present study, it is not clear whether TGF-β1- or other growth factor-primed ES cells will release similar factors.

**Fig. 5.** Heart sections were stained with Masson’s trichrome to determine fibrosis. A: representative photomicrographs of cardiac fibrosis. Sham-treated animals show no blue stain, indicating the absence of fibrosis (a); blue area indicates fibrosis in MI animals (b); and less blue area is seen in MI+ES-CM (c) and MI+T-ES-CM (d) animals. B: quantitation of cardiac fibrosis. Data are from a set of 6–8 different animals. *P < 0.05 vs. MI; #P < 0.05 vs. ES-CM.
Before investigation in the microenvironment of an animal model, we developed the cell culture model system described above in order to simulate the post-MI heart. This model allows us to efficiently and rapidly examine a variety of different conditions that are influenced by released factors in CM. Therefore, our present findings suggest that T-ES-CM contains increased amounts of cytoprotective released factors, which offers enhanced protection from apoptotic cell death in the two independent models of oxidative stress-induced apoptosis in the cell culture model. As already established, apoptosis is significantly increased in various models of cardiovascular diseases including MI (2, 3, 35). However, whether increased amounts of cytoprotective factors in the T-ES-CM will also inhibit significant apoptosis compared with unprimed ES-CM in the infarcted heart remains largely unknown. We hypothesize that TGF-β2-primed ES-CM after transplantation in the infarcted heart will inhibit increased amounts of apoptosis and improve cardiac function. In the present study, our data suggest that T-ES-CM significantly inhibits apoptosis and fibrosis compared with ES-CM after post-MI transplantation at D14.

Cell survival pathways such as Akt and ERK are major players in the oxidative stress-induced apoptosis in cell culture and heart injury models (1, 7, 19, 39). We reported (39) that the Akt protein is significantly increased in H2O2-induced apoptosis in H9c2 cells (19) and HL-1 cardiomyocytes (6), as well as in reperfusion injury (25). In the present study, we have shown that pAkt enhances cell survival in the infarcted heart after treatment with ES-CM or T-ES-CM. Moreover, our data also suggest that decreased apoptosis in the infarcted heart following treatment with ES-CM or T-ES-CM. Moreover, our data also suggest that decreased apoptosis in the infarcted heart following treatment with ES-CM or T-ES-CM. Moreover, our data also suggest that decreased apoptosis in the infarcted heart following treatment with ES-CM or T-ES-CM. Moreover, our data also suggest that decreased apoptosis in the infarcted heart following treatment with ES-CM or T-ES-CM. 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Table 1. Antiapoptotic factors released in TGF-β2-treated and untreated ES cell CM

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<tr>
<th>Released Factors</th>
<th>Control</th>
<th>ES-CM</th>
<th>T-ES-CM</th>
<th>Fold Increase in T-ES-CM vs. ES-CM</th>
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<tbody>
<tr>
<td>IL-10</td>
<td>L</td>
<td>27</td>
<td>48</td>
<td>1.5</td>
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<tr>
<td>SCF</td>
<td>L</td>
<td>9</td>
<td>20</td>
<td>2</td>
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<tr>
<td>VEGF</td>
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<td>845</td>
<td>5,040</td>
<td>6</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>8.4</td>
<td>1,200</td>
<td>5,000</td>
<td>4</td>
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</table>

Values are expressed as pg/ml. ES, embryonic stem; TGF-β2, transforming growth factor-β2; CM, conditioned medium; T-ES-CM, TGF-β2-primed ES-CM; Control, cell culture medium used to grow ES cells; L, lowest dose detectable by the assay (see MATERIALS AND METHODS); IL-10, interleukin-10; SCF, stem cell factor; VEGF, vascular endothelial growth factor; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.
apoptotic in H9c2 cells, PC-12 cells, endothelial cells, cancer cells, and neuroblastoma cells (20, 22, 29, 31, 32, 43). In the present study, we have shown that TGF-β2-primed released factors have additional beneficial effects compared with unprimed ES-CM on IAA- and H2O2-induced apoptosis. We suggest that the additional beneficial cytoprotective effects of T-ES-CM are obtained from all four factors reported. However, the question of whether all four released factors examined in the present study have equal impact on cell protection in vitro and in vivo needs further investigation. Our findings suggest that factors released from ES cells are unique and their effects are selective for ES cells, as we have reported previously (38) that CM prepared from H9c2 cells does not confer protective effects are selective for ES cells, as we have reported previously (38) that CM prepared from H9c2 cells does not confer protection in oxidative stress-induced apoptosis. Thus our data generated in the apoptotic models of ischemic insult and protection in oxidative stress-induced apoptosis. Therefore, our data previously (38) that CM prepared from H9c2 cells does not confer protection in oxidative stress-induced apoptosis. Thus our data suggest that T-ES-CM significantly inhibits apoptosis compared with respective controls.

In summary, we suggest that ES cells treated with TGF-β2 release factors that contain antiapoptotic factors and provide cytoprotective effects against IAA- and H2O2-induced cell death in vitro, as well as after transplantation in the infarcted heart. However, further effects of released factors from T-ES-CM following transplantation in the infarcted heart require elucidation to identify potential signaling mechanisms that may have major implications in regenerative medicine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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