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Embryonic Stem Cells Improve Cardiac Function in Doxorubicin-Induced Cardiomyopathy Mediated Through Multiple Mechanisms

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Doxorubicin (DOX) is an effective antineoplastic agent used for the treatment of a variety of cancers. Unfortunately, its use is limited as this drug induces cardiotoxicity and heart failure as a side effect. There is no report that describes whether transplanted embryonic stem (ES) cells or their conditioned medium (CM) in DOX-induced cardiomyopathy (DIC) can repair and regenerate myocardium. Therefore, we transplanted ES cells or CM in DIC to examine apoptosis, fibrosis, cytoplasmic vacuolization, and myofibrillar loss and their associated Akt and ERK pathway. Moreover, we also determined activation of endogenous c-kit^{+ve} cardiac stem cells (CSCs), levels of HGF and IGF-1, growth factors required for c-kit cell activation, and their differentiation into cardiac myocytes, which also contributes in cardiac regeneration and improved heart function. We generated DIC in C57Bl/6 mice (cumulative dose of DOX 12 mg/kg body weight, IP), and animals were treated with ES cells, CM, or cell culture medium in controls. Two weeks post-DIC, ES cells or CM transplanted hearts showed a significant ($p<0.05$) decrease in cardiac apoptotic nuclei and their regulation with Akt and ERK pathway. Cardiac fibrosis observed in the ES cell or CM groups was significantly less compared with DOX and cell culture medium groups ($p<0.05$). Next, cytoplasmic vacuolization and myofibrillar loss was reduced ($p<0.05$) following treatment with ES cells or CM. Moreover, our data also demonstrated increased levels of c-kit^{+ve} CSCs in ES cells or CM hearts and differentiated cardiac myocytes from these CSCs, suggesting endogenous cardiac regeneration. Importantly, the levels of HFG and IGF-1 were significantly increased in ES cells or CM transplanted hearts. In conclusion, we reported that transplanted ES cells or CM in DIC hearts significantly decreases various adverse pathological mechanisms as well as enhances cardiac regeneration that effectively contributes to improved heart function.

Key words: Stem cells; Heart; Doxorubicin (DOX); Apoptosis; Fibrosis

INTRODUCTION

Doxorubicin (DOX) is an antineoplastic antibiotic vastly used antitumor agent for a diversity of malignancies (22,27). However, the clinical use of this drug is restricted due to a severe, dose-dependent, acute cardiotoxicity that may progress to irreversible chronic cardiomyopathy and congestive heart failure. DOX-induced cardiomyopathy (DIC) has been well published in human and animal studies. Even though, the precise mechanism of DIC is unclear, antitumor activity of DOX is relayed to be distinct from the mechanisms of induced cardiomyopathy. DIC appears to involve multifactorial and complex disease mechanisms; however, oxidative stress plays a major role in this cardiotoxicity. DIC is characterized by contractile dysfunction and rhythm disturbances that lead to congestive heart failure in a time-dependent manner (22,27). Moreover, development of heart failure includes (a) death of both cardiac myocytes and nonmyocyte myocardial cells, (b) myofibril loss and vacuolar degeneration, and (c) fibrosis. These changes result in rearrangement of heart tissue, increased wall stress, and insufficient systolic contractility in cardiac myocytes (12,22,27,38). Importantly, the collagen synthesis that is increased in DIC is also associated with concurrent extracellular matrix (ECM) degradation via activation of matrix metalloproteinases (MMPs) (12,33,38). Numerous studies have demonstrated inhibiting doxorubicin-induced cardiac myocyte apoptosis in DIC using various agents such as erythropoietin and antioxidants (11–13). However, DIC is a major health problem; therefore, accurate identification of novel therapeutic approaches is still warranted.

Over the past decade, cell transplantation studies have demonstrated significant interest as a potential option to treat distinct heart diseases including DIC (1,9,39). Published studies demonstrate significant improvement in cardiac function in DIC following adult stem cell transplantation (1,9,39). Moreover, these studies proclaimed minimal or no

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successful engraftment of transplanted cells (1,39). There is no data yet attainable that describes the ability of embryonic stem (ES) cells or their factors released to repair and regenerate DIC; however, ES cells have a distinct advantage as they are unique in the potential to differentiate into many body cell types compared with their counterpart adult stem cells (28). Therefore, in the present study, we hypothesized that transplanted ES cells or their conditioned medium (CM), containing cytoprotective factors, will inhibit DIC. We present data in this study on transplanted ES cells or CM that inhibit cardiac apoptosis, fibrosis, cytoplasmic vacuolization, and myofibril loss, typical characteristics of DIC. Additionally, we also determined significant increased levels of hepatocyte growth factor (HGF) and insulin growth factor (IGF-1) in hearts transplanted with ES cells or CM, required in activating c-kit^{+ve} cardiac stem cells (CSCs). Moreover, increased numbers of c-kit^{+ve} CSCs were predominantly observed in the same HGF and IGF-1 hearts. Finally, we observed significant improvement in cardiac function following ES cells or CM transplantation, suggesting multiple mechanisms are required to blunt DIC.

MATERIALS AND METHODS

ES Cells and Preparation of CM

Mouse ES cells expressing green fluorescence protein (GFP) were maintained using our standard protocol as previously described (30,32). In brief, cells were plated 24 h on gelatinized tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) containing essential ingredients such as fetal bovine serum (FBS), leukemia inhibitory factor (LIF), glutamine, penicillin/streptomycin, sodium pyruvate, and β-mercaptoethanol. For ES-CM, cell culture medium was replaced with cell culture medium without LIF. After 48 h, released factors containing cell supernatant was removed, filtered (0.2-μm filter, Millipore, USA), and used to inject sterile CM as we previously published (30,32).

Animals and Experimental Protocol

All the animals described in this study are approved by University of Central Florida animal care committee. C57Bl6 mice were divided into five different study groups: control, normal saline, DOX, DOX+cell culture (CC) medium, DOX+ES cells, or DOX+CM. There were $n=6$–8 animals in each of the five study groups.

DOX was administered in three equal injections in mice (4 mg/kg/injection IP) on alternate days over the period of 1 week for a cumulative dose of 12 mg/kg. For treatment groups, CC medium (400 μl/injection and the total of three injections), ES cells (5×10^5 ES cells/injection and total of three injections), or ES-CM (400 μl/injection and total of three injections) was injected during DOX treatment on alternative days. For example, DOX or normal saline in controls 400 μl/injection was given on Monday, Wednesday, and Friday, whereas treatment groups, CC medium, ES cells, or ES-CM were given on Tuesday, Thursday, and Saturday. After the last injection of treatment groups, animals were examined for heart function using echocardiography and were sacrificed at day (D) 14 by administering pentabarbitol (40 mg/kg, IP). Furthermore, half of the heart tissue was fixed in 4% paraformaldehye, whereas the other half was saved in RNA later to perform biochemical and ELISA assays.

Identification of Apoptotic Nuclei and Caspase-3 Staining

Apoptotic nuclei in heart sections with or without cell transplantation groups were identified using a commercially available terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay kit; TMR red; Roche Applied Biosystems, IN, USA) as per detailed instructions available with the kit. Moreover, we have published this protocol in our previous studies (29). Next, we identified colabeling of TUNEL-positive apoptotic nuclei with proapoptotic caspase-3 as well as with sarcomeric cardiac α-actin to determine if apoptosis does occur in cardiac myocytes. In brief, TUNEL-stained sections were stained with primary antibodies; active anti-caspase-3 rabbit polyclonal (1:1,000; Cell Signaling) and sarcomeric cardiac α-actin mouse monoclonal antibodies (1:30; Sigma). Following incubations, sections were washed with PBS and labeled with secondary antibodies: anti-rabbit Alexa 568 (Invitrogen) or anti-mouse Alexa 488 (MOM kit, vector laboratories). Total nuclei were stained blue with DAPI (4′,6-diamidino-2-phenylindole) present in the mounting Vectashield medium. Positively stained cells were examined, and photomicrographs were prepared with an Olympus fluorescence microscope and a LEICA laser scanning confocal microscope. Red stained apoptotic nuclei present in the left ventricular area of the heart were examined, and quantifications were determined by two blind observers in four to five random areas in one to two heart sections from six to eight different hearts as published (29). To determine percentage of apoptotic nuclei per section, we calculated using the formula: number of apoptotic nuclei/total number of nuclei per section and then multiply by 100. Percentage of apoptotic nuclei data was examined under 20× magnification.

Caspase-3 Activity

Heart homogenates were prepared from different DOX and ES cell-treated groups. Caspase-3 activity was performed using a caspase-3 colorimetric activity assay kit from BioVision (CITY, CA, USA) as we reported recently (5). Protein concentrations were measured in

the supernatant using a Bio-Rad assay. Caspase-3 activity was performed as per the instructions provided in the kit and measured at 405 nm in a microtiter plate reader. Caspase-3 activity was calculated and plotted in the form of arbitrary units (A.U.).

Phosphorylated Akt Activity

Phosphorylated Akt (p-Akt) and phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK 1/2) activities were examined using commercially available kits (Exalpha Biologicals, Inc., Maynard, MA). In brief, tissues were homogenized as described beforehand, and p-Akt and p-ERK 1/2 were quantified according to manufacturer's instructions. The reactions were developed, and the color reactions were measured at 450 nm using a microtiter plate reader for each ELISA. p-Akt and p-ERK 1/2 protein data were plotted as ng/mg tissue and arbitrary units (A.U.), respectively.

Determination of HGF and IGF-1 in Heart Tissue in DIC

Hearts were isolated; homogenates were prepared with the assay buffer provided with the kit. Using our standard Bio-Rad assay, total protein concentrations were determined. Commercially available kits, HGF (B-Bridge International, CA) and IGF-1 (R&D, Minneapolis, USA), were purchased and used to measure HGF and IGF-1 levels in the DIC hearts with or without treatment groups. These kits are based on the ELISA sandwich assay, which was performed in accordance with the kit instructions. HGF and IGF-1 concentrations in the samples were analyzed from the standard curve performed according to the kit directions.

Determination of Myofibrillar Loss, Cytoplasmic Vacuolization, and Fibrosis

Hearts were rapidly removed, placed in iced saline, fixed in 4% buffered formalin and embedded in paraffin as described previously (29). 5-μm serial sections were cut and deparaffinized by incubation in xylene. Rehydration was accomplished by sequential incubation in 100%, 95%, and 70% ethanol for 5 min each at room temperature, followed by washing in distilled water and then phosphate-buffered saline (PBS) for 5–10 min. Heart sections were stained with commonly used H&E and Mason's trichrome protocols. Two to three H&E-stained heart sections were examined completely for the evaluation of myofibrillar loss and cytoplasmic vacuolization. Qualitative data scale, developed to determine cytoplasmic vacuolization and myofibrillar loss, varies from 0 to 2. If left ventricular (LV) heart section showed no signs of myofibrillar loss and cytoplasmic vacuolization then score was graded as 0, whereas if significant portion of the section had myofibrillar loss and cytoplasmic vacuolization then slide was graded as 2. To determine cardiac fibrosis, sections were stained with Masson's trichrome. Cardiac fibrosis predominantly present in the left ventricle was examined and quantified by measuring the total blue area per mm^2 in each section using freely available ImageJ, NIH program.

Identification of c-Kit^{+ve} Cells and Their Differentiation Into Cardiac Myocytes

Sections were deparaffinized as described above. To block nonspecific staining, sections were incubated in humidified chamber in 10% normal goat serum (NGS) from 30 min to 1 h at room temperature. Following incubation, sections were washed and incubated with primary rabbit polyclonal antibody anti-c-kit (Santa Cruz Biotechnologies) and sarcmomeric cardiac α-actin mouse monoclonal antibody (Sigma), diluted with 10% NGS, for an hour at 37°C. Sections were washed in PBS and incubated with secondary antibody Alexa Fluor 568 or antimouse fluorescein isothiocyanate (FITC) available in the MOM kit (Vector Laboratories) for 1 h. Heart sections were washed and mounted with Vectashield antifade medium (containing nuclear stain DAPI; Vector Laboratories). Quantitative analysis of c-kit^{+ve} cells was determined on heart sections from four to six different hearts using Olympus and confocal fluorescence microscope.

Echocardiographic Analysis

We routinely performed 2D echocardiography on mice using a Sonos 5500 ultrasonograph with a 15-MHz transducer (Philips, Andover, MA). Mice were sedated with ketamine (100 mg/kg, IM), the chest was shaved and maintained on a heated platform to control temperature, and placed in a supine or left lateral decubitus position to measure left ventricular (LV) functions using 2D echocardiography. For quantification of LV dimensions and wall thickness, we digitally recorded 2D clips and M-mode images in a short axis view from the mid-LV at the tips of the papillary muscles. End diastolic and systolic LV diameter, as well as anterior and posterior wall (AW and PW, respectively) thickness, was measured on M-mode images using the leading edge-to-leading edge convention. LV fractional shortening and mass were calculated from LV cross-sectional area in 2D short axis view as $[(LVD_{diastole} - LVD_{systole} / LVD_{diastole})] \times 100$ and LV mass with formula $[1.05 \times (PW_{diastole} + AW_{diastole} + LVD_{diastole})^3 - (LVD_{diastole})^3]$. Relative wall thickness was calculated as $2 \times PW_{diastole} / LVD_{diastole}$.

Data Analysis

Values were presented as means ± SEM. One-way analysis of variance (ANOVA) was performed followed by Dunn's test. Differences between values were considered statisticaly significant when $p < 0.05$.

RESULTS

Effects of Transplanted ES Cells or CM on Apoptosis in DIC

Figure 1 (A–O) shows apoptotic positive nuclei with TUNEL staining. Right panel histogram shows there was a significant ($p < 0.05$) increase in TUNEL-positive nuclei in DOX and DOX + CC medium-treated hearts compared with normal saline controls. This significant increase in apoptotic nuclei were attenuated in DOX + CM or DOX + ES cell groups, suggesting protective effects of CM or ES cell treatments ($p < 0.05$) (Fig. 1P). Next, we wanted to investigate whether apoptotic nuclei are present in the cardiac myocytes as well as stained positive with active caspase-3 staining, considered as a hallmark of apoptosis. Therefore, heart sections from all groups were stained with TUNEL, colabeled with sarcomeric cardiac α-actin and active caspase-3 antibody. Figure 2 (A–E) shows that TUNEL-stained nuclei were positive with active caspase-3 as well as suggesting apoptosis does occur in cardiac myocytes as confirmed with sarcomeric cardiac α-actin. Moreover, we performed a caspase-3 activity assay as shown in Figure 2F. Significantly increased caspase-3 activity in DOX and DOX + CC hearts compared with controls were attenuated following CM or ES cell transplantation. However, apoptosis and caspase-3 activities were not significant between DOX + CM and DOX + ES cell groups.

CM or ES Cells Regulate Akt and ERK Pathways in DIC

Apoptosis has been regulated with increased or decreased expressions of Akt and ERK signaling pathways in ischemic heart diseases (8,17,32). Therefore, we examined effects of these pathways in the DIC and their modulation with transplanted CM or ES cells. Phosphorylated Akt was significantly reduced in the DOX and DOX + CC groups compared with normal controls, suggesting a decrease in cell survival. Moreover, hearts transplanted with CM or ES cells had significant increased activation of p-Akt compared to DOX and DOX + CC (Fig. 3A). Moreover, our data suggest phosphorylated ERK1/2 is significantly increased in DOX and DOX + CC groups compared with control. A significant decrease in ERK1/2 was observed in hearts transplanted with CM or ES cells ($p < 0.05$) (Fig. 3B).

CM or ES Cells Inhibit Fibrosis, Cytoplasmic Vacuolization, and Myofibrillar Loss

To determine the effect of transplanted CM or ES cells on cardiac fibrosis, we stained heart sections with Masson's trichrome. In Figure 4 (A–E), the blue areas show fibrotic tissue present in the DOX-treated hearts with or without CM or ES cells. Our quantitative data suggest that DOX and DOX + CC hearts demonstrated a significant ($p < 0.05$) increase in blue area, an indicative of cardiac fibrosis (Fig. 4F). However, this increased fibrosis was reduced following CM or ES cell transplantation

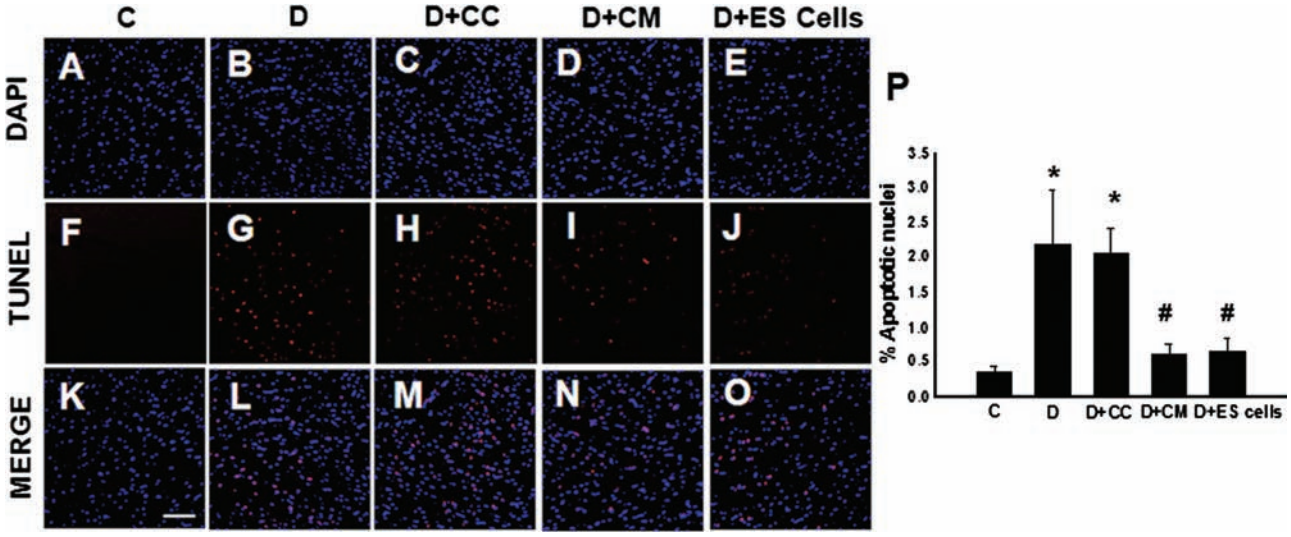

Figure 1. Transplanted conditioned medium (CM) or embryonic stem (ES) cells inhibit cardiac myocyte apoptosis at 2 weeks post-doxorubicin-induced cardiomyopathy (DIC). Representative photomicrographs of total nuclei stained with DAPI in blue (A–E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-stained apoptotic nuclei in red (F–J) and merged nuclei in pink (K–O); scale bar: 50 μm. Right: histogram (P) shows quantitative apoptotic nuclei per section from control (C), DOX (D), D + culture medium (D + CC), D + CM, and D + ES cell groups. Data are from the $n = 6$–9 animals (*$p < 0.05$ compared to C; #$p < 0.05$ vs. D and D + CC).

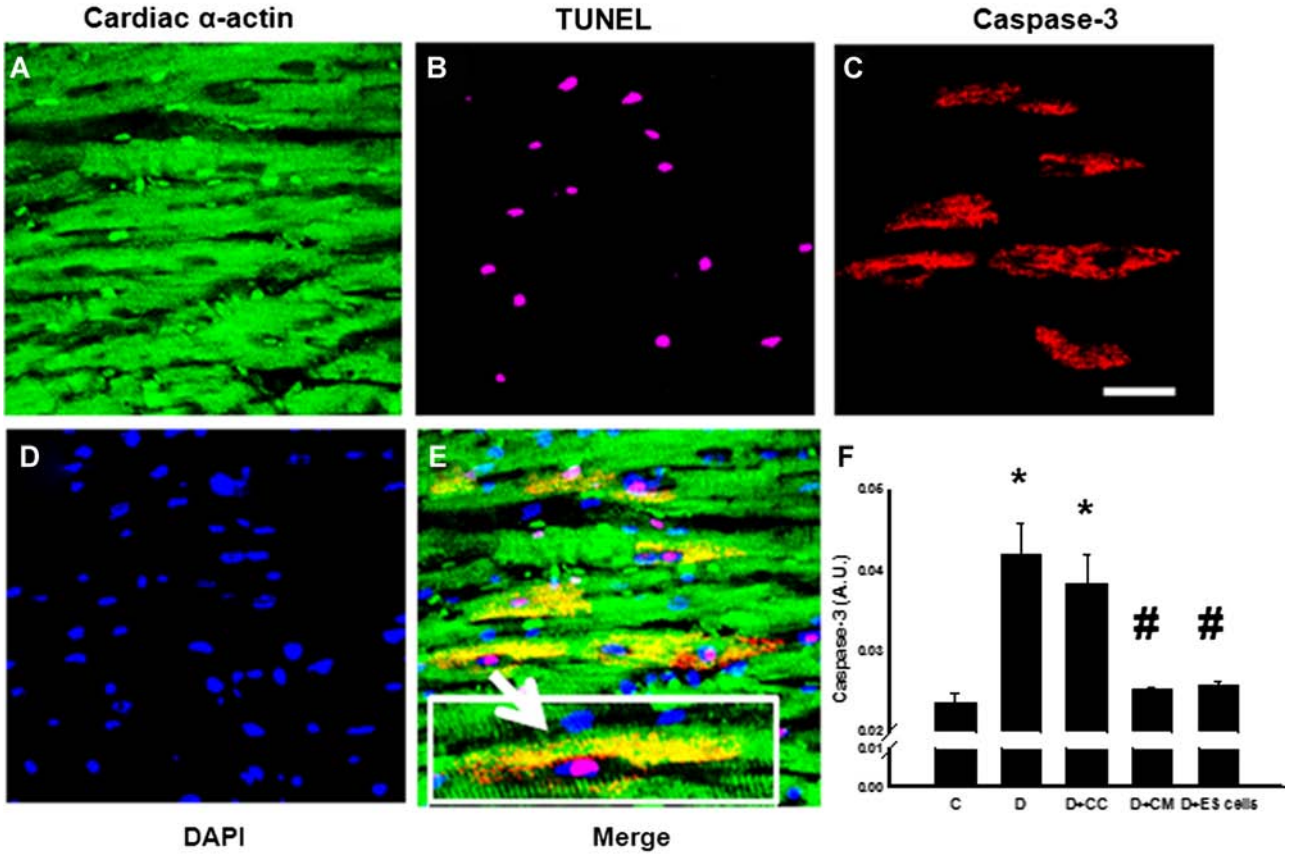

Figure 2. Effects of transplanted CM or ES cell on cardiac myocyte apoptosis and caspase-3 activity. Representative photos of heart section labeled with sarcomeric cardiac α-actin (A), apoptotic nuclei stained with TUNEL in purple (B), section stained with active caspase-3 antibody (C), total nuclei stained with DAPI in blue (D), and merged image (E) demonstrating apoptosis appearing in cardiac myocytes and positive with active caspase-3. Boxed area in (E), demonstrates colocalization of sarcomeric cardiac α-actin, active caspase-3 (yellow area), TUNEL-positive nucleus, and DAPI. Scale bar: 25 μm. Bottom right histogram (F) shows total apoptotic nuclei from five to eight different animals each group (*$p < 0.001$ vs. C, #$p < 0.05$ vs. D and D + CC). A.U., arbitrary units.

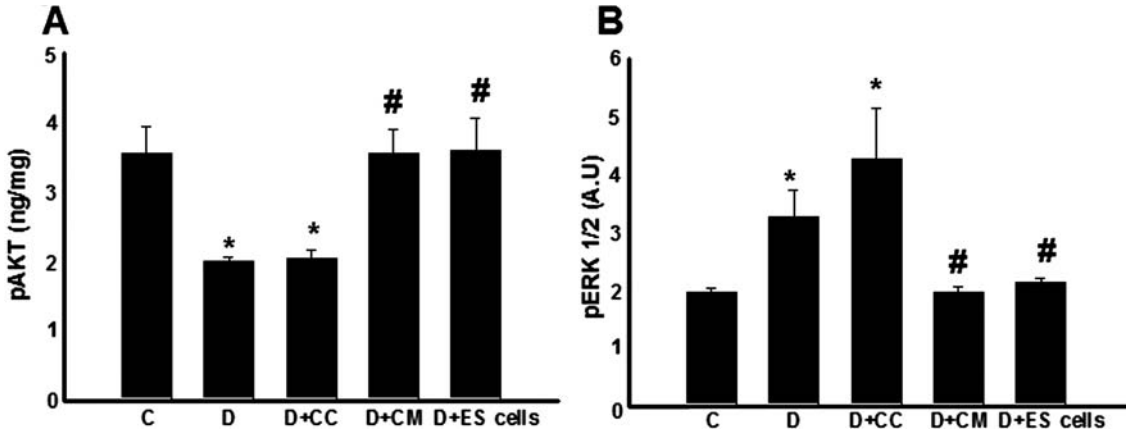

Figure 3. Phospho-Akt and extracellular signal regulated kinase (ERK) alterations following CM or ES cell transplantation in the DIC. (A) Performed quantitative analysis of phospho-Akt regulation. (B) Quantitative levels of phosphorylated ERK upregulation (*$p < 0.05$ compared to C; #$p < 0.05$ vs. D and D + CC). Data are from heart homogenates of $n = 4$–6 animals/group. A.U., arbitrary units.

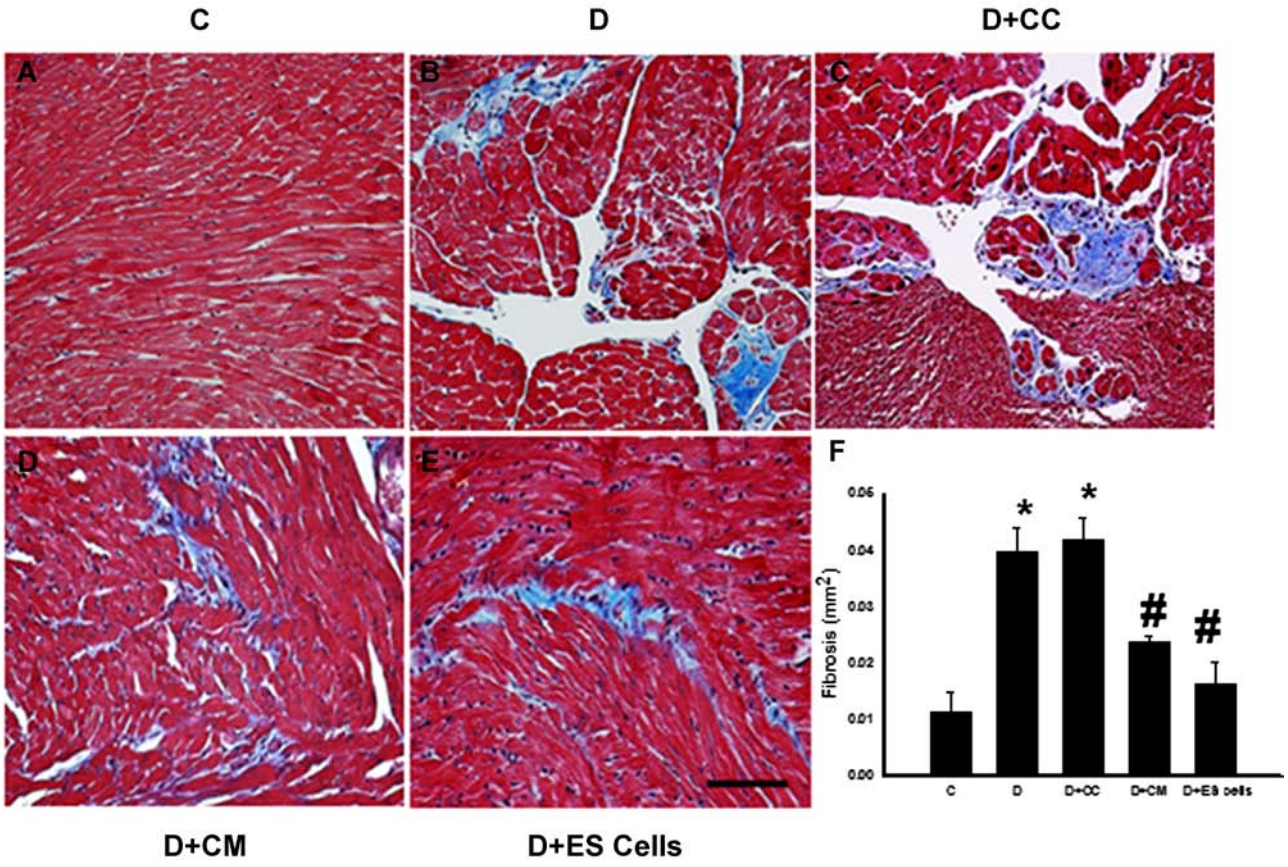

Figure 4. Effects of transplanted CM or ES cell on cardiac fibrosis. Photomicrographs show histological sections stained with Mason's trichrome after 2 weeks of DOX treatment of different hearts from each group. Blue area shows extent of fibrosis in different conditions: control, C (A); DOX, D (B); D+CC (C); D+CM (D); and D+ES cells (E). Scale bar: 50 μm. (F) Histogram shows quantitative cardiac fibrosis in post-DIC with or without treatment groups. Data are from six to eight animals (*$p<0.05$ compare to C; #$p<0.05$ vs. D and D+CC).

(Fig. 4F). Therefore, relative increased amounts of viable cardiac muscle were observed in the DOX+CM or ES cells (Fig. 4D, E) compared with DOX or DOX+CC groups (Fig. 4B, C).

Cyoplasmic vacuolization and myofibrillar loss, a well-recognized characteristic of DIC, were examined to strengthen our findings. In normal saline-treated control, hearts show full-length cardiac myocytes with intact cytoplasmic structure as well as well-organized myofibrillars (Fig. 5A). In contrast, DOX- or DOX+CC-treated hearts demonstrate clear evidence of loss of cytoplasm as well as myofibrils in many cardiac myocytes (Fig. 5B, C), and this increased cytoplasmic vacuolization and myofibrillar loss was significantly reduced following CM or ES cells treatments (Fig. 5D, E). Moreover, our semiquantitative data suggest significant ($p<0.05$) increase in cytoplasmic vacuolization and myofibrillar loss in DOX and DOX+CC hearts (Fig. 5F, G). Following treatments with CM or ES cells in DIC, hearts showed significantly reduced amounts of cytoplasmic vacuolization and myofibrillar, suggesting a decrease in cardiomyopathy (Fig. 5F, G).

c-Kit^{+ve} Cells and Their Progenitor Cells Following CM or ES Cell Transplantation

To determine activated cardiac endogenous c-kit^{+ve} cells and their differentiation into cardiac myocytes to define their role in cardiac regeneration, we examined c-kit^{+ve} cells and their colabeling with sarcomeric cardiac α-actin to stain cardiac myocytes in heart sections with or without CM or ES cell transplantation in DIC. Figure 6 (A–D) shows positively stained c-kit^{+ve} cells as well as c-kit^{+ve} cells combined with cardiac myocyte-specific sarcomeric cardiac α-actin, suggesting some of the c-kit^{+ve} cells differentiate into cardiac myocytes. Quantitative c-kit^{+ve} cells in DOX or DOX+CC significantly increased ($p<0.05$) compared with normal saline controls, suggesting injury released cytokine

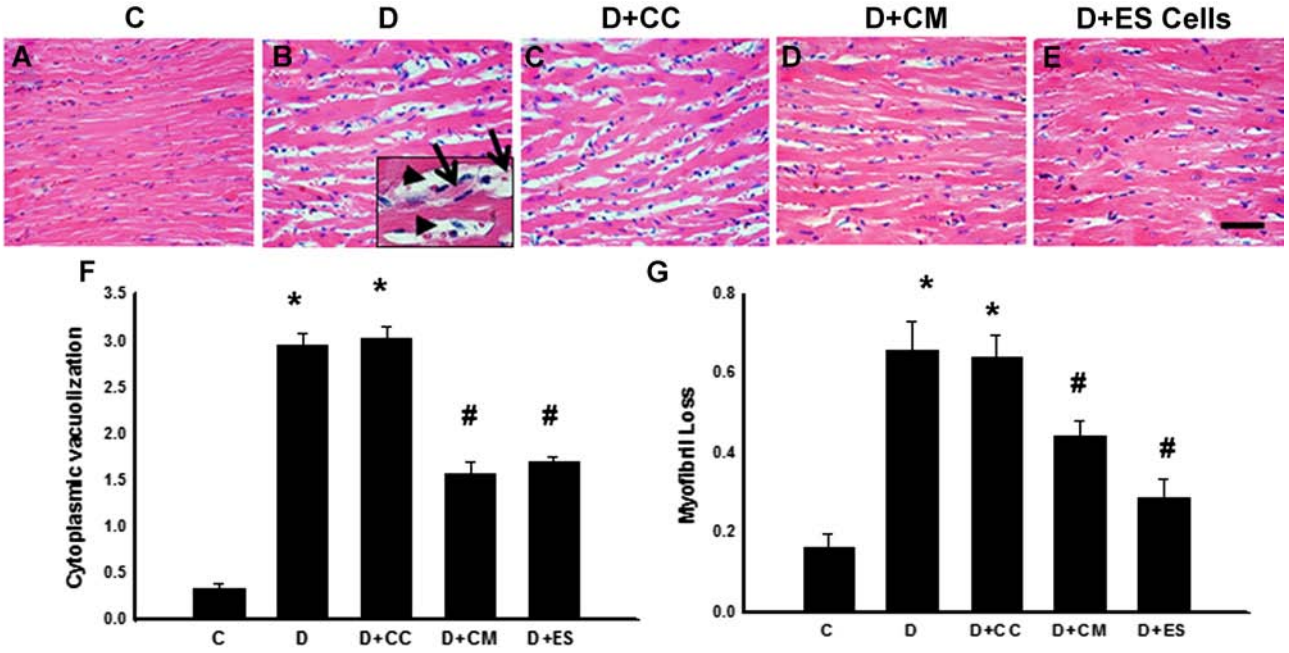

Figure 5. Effects of transplanted CM or ES cell on cytoplasmic vacuolization and myofibrillar loss. Photomicrographs show histological sections stained with H&E staining after 2 weeks of DOX treatment of different hearts from each group. In the representative enlarged area, the arrow head indicates cytoplasmic vacuolization whereas the arrow shows myofibrillar loss. These cytoplasmic vacuolization and myofibrillar loss areas in cardiac myocytes were determined in different conditions: C (A), D (B), D+CC (C), D+CM (D), and D+ES cells (E). Scale bar: 50 μm. (F) Histogram shows semiquantitative cytoplasmic vacuolization. (G) Determines myofibrillar loss in post-DIC with or without treatment groups. Data are from five to eight animals (*$p<0.05$ compare to C; #$p<0.05$ vs. D and D+CC).

and growth factors may have triggered c-kit^{+ve} cells (Fig. 6E). Moreover, c-kit^{+ve} cells were further significantly increased in CM or ES cells groups compared with respective controls (Fig. 6E).

Levels of HGF and IGF-1 Following CM or ES Cells Transplantation

Following an increase in c-kit^{+ve} cells in CM or ES cell groups in DIC, we determined the levels of HGF and IGF-1, a growth factor considered to be an activator of c-kit^{+ve} cells. Our data demonstrate that heart tissue homogenates prepared from CM- or ES cell-treated hearts have significant increased levels of HGF and IGF-1 (Fig. 6F, G). Thus, increased HGF and IGF-1 levels suggest that these factors released into DIC hearts following CM or ES cell transplantation may also contribute to the stimulation and differentiation of the CSCs that play a role in regeneration.

No Evidence of Teratoma Formation Following ES Cell Transplantation in DOX Cardiomyopathy

To determine whether teratoma were present following ES cell transplantation, H&E and Masson's trichrome-stained heart, lung, and liver sections were examined at 2 weeks post-DOX treatment. No evidence of teratoma formation was observed at 2 weeks in the heart, lung, and liver. Figure 7 is representative of these sections at 2 weeks post-DOX-treated hearts.

Improvement in LV Function Following CM or ES Cells Transplantation

M-mode echocardiography was used to determine the effect of CM or cell transplantation on LV size and function in mice at 2 weeks post-DOX treatment. Fractional shortening was improved by CM and ES cell transplanted hearts compared with DOX or DOX+CC groups (Fig. 8A). Additionally, CM and ES cells also demonstrated significantly improved ejection fraction (Fig. 8B). Moreover, the increased LVIDs in DOX and DOX+CC groups was significantly ($p<0.05$) (Fig. 8C) blunted following CM or ES cell transplantation.

DISCUSSION

Cell therapy has been widely examined in various animal and human cardiac diseases (9,15,16,20,21,24,25). Animal studies have successfully manifested significant improvement in cardiac function (24,25). Human embryonic stem cell-derived vascular cells and bone marrow-derived multipotent progenitor cells following transplantation in the porcine infarcted heart improved cardiac function (36,37).

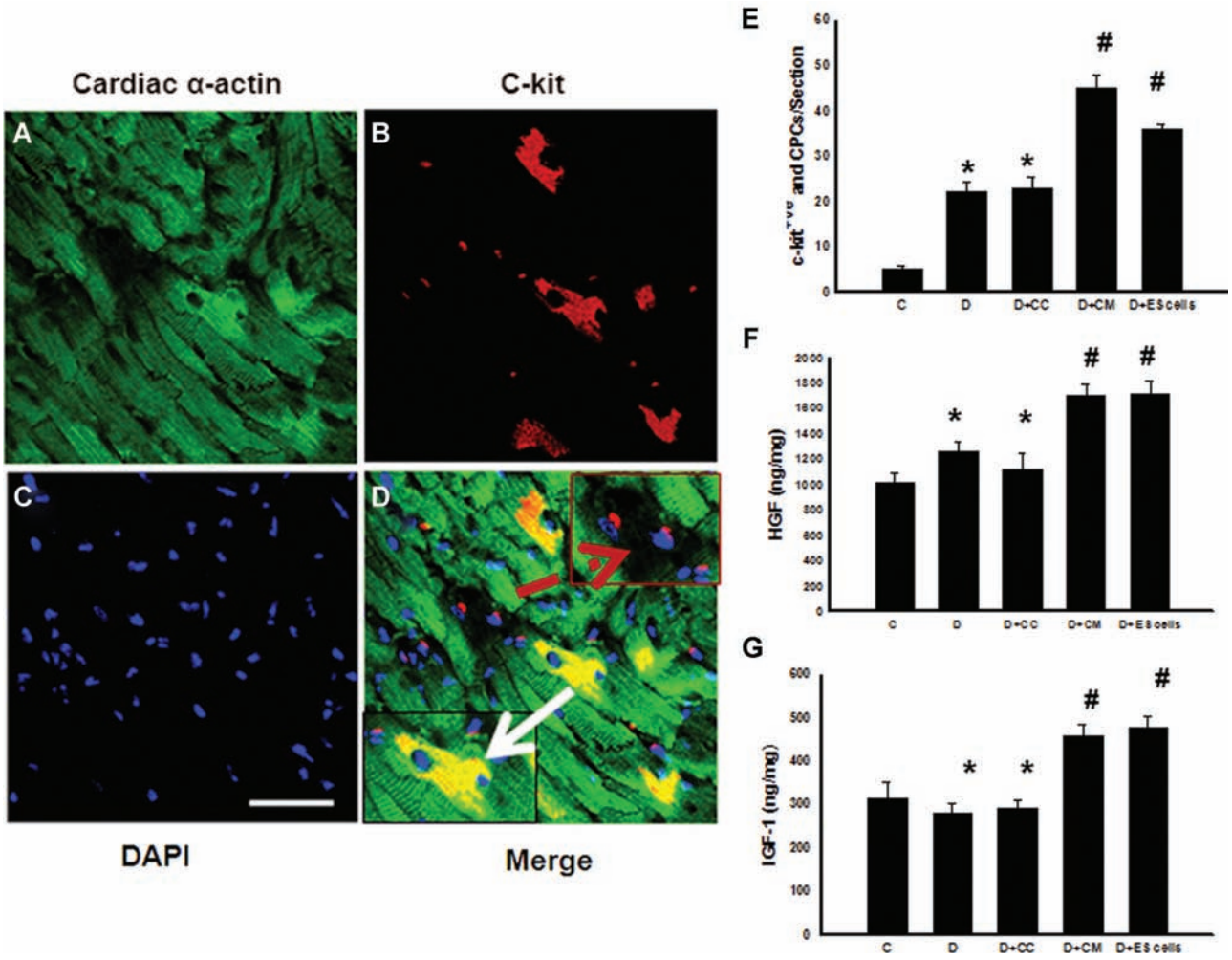

Figure 6. Effects of transplanted CM or ES cell on cardiac stem cells (CSCs) and their differentiation into cardiac myocytes. Heart section stained with sarcomeric cardiac α-actin showing all stained cardiac myocytes in green (A), resident CSCs were identified by anti-c-kit antibody labeling in red (B), and nuclei are stained with DAPI in blue (C). Merged image indicating presence of CSCs and the positive staining with cardiac α-actin (D, green, red, and blue). Bottom left arrow indicates differentiated CSCs, whereas dashed arrow in right top panel shows CSCs positive with c-kit staining. Scale bar: 50 μm. (E) Histogram shows quantitative cardiac progenitor cells in post-DIC hearts from C, D, D + CC, D + CM, and D + ES cells groups. Data are from four to five animals (*$p < 0.05$ compared to C; #$p < 0.05$ vs. D and D + CC). (F) Histogram shows hepatocyte growth factor (HGF) data analysis performed in different DIC groups. (G) Histogram shows levels of insulin-like growth factor (IGF)-1 in the DIC hearts with or without treatment groups. Data are from four to five animals (*p is nonsignificant compared to C; #$p < 0.05$ vs. D and D + CC).

Moreover, bone marrow-derived stem cells or combined therapy with cardiomyocytes inhibits doxorubicin-induced heart failure in rabbits (7,15,16). Unfortunately, adult stem cell transplanted clinical studies provided contentious reports on the improvement of cardiac function (9,21). Therefore, the use of ES cells may be an optimal cell type, with a wide potential for cell differentiation and unique in characteristics as released factors from these cell types are quite different compared with adult stem cells. Moreover, there is no data yet available that determines the effects of CM or ES cells on DIC. Therefore, we hypothesize that transplanted CM or ES cells will inhibit multiple pathological mechanisms in DIC and will improve associated cardiac function.

In the present study, we used a very clinical relevant DIC cardiomyopathy model, which is well established by various investigators (4,26,27). Importantly, DOX as administered in the present study demonstrates cardiac apoptosis, regulation of apoptotic-mediated Akt/ERK pathways, cardiac fibrosis, cytoplasmic vacuolization, myofibrillar loss, and decrease in cardiac function in terms of fractional shortening and ejection fraction as deliberated by echocardiography. These alterations construct DIC as observed in cancer patients treated with DOX (4,26,27).

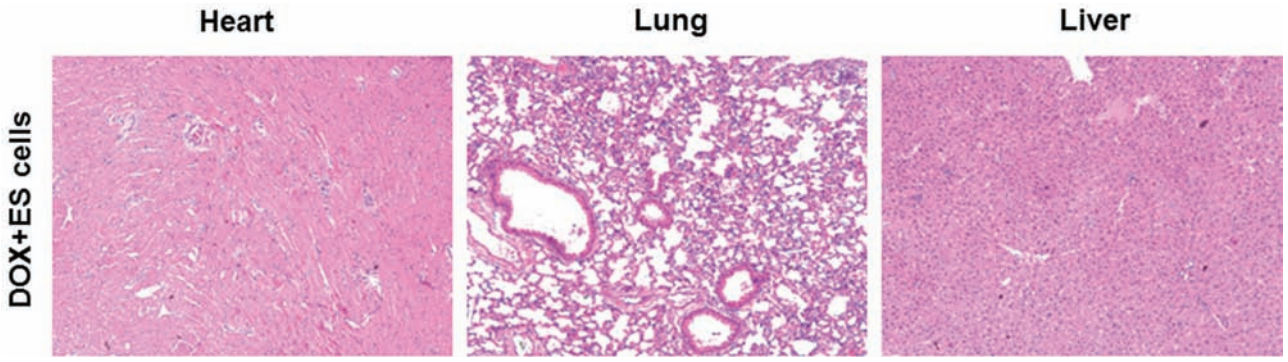

Figure 7. Representative photomicrographs of H&E-stained heart sections from DOX+ES cell group shows no evidence of teratoma formation in the heart, lung, and liver post-DIC at 2 weeks. Original magnification: 10×.

Therefore, we report that the DIC model in the present study is in accordance with published reports (4,26,27). The major findings of the present study is that transplantation of the CM or ES cells in DIC results in significant attenuation of various pathological mechanisms such as (1) inhibition of cardiac myocyte apoptosis, (2) inhibited apoptosis involving phosphoinositol-3-kinase (PI3K)/Akt and ERK pathways, (3) significantly attenuated cardiac fibrosis, (4) inhibition of cytoplasmic vacuolization and myofibrillar loss, (5) activation of c-kit^{+ve} CSCs and their role in cardiac regeneration, (6) increased levels HGF and IGF-1 growth factors required for c-kit^{+ve} cells activation, and (7) ultimately improved cardiac function.

Apoptosis is an elemental part of normal organ development (2,14). Recently, presence of apoptosis has been notified to regulate various diseases, including DIC (2, 13,14). We and others have reported that apoptosis is a significant contributor in the development and progression of DIC (4,13,14,22). The data on apoptosis in this investigation were established with TUNEL staining as well as by measuring caspase-3 activities. We also demonstrate that hearts transplanted with CM or ES cells have a significantly reduced amount of apoptosis. Additionally, we show that apoptosis does occur in cardiac myocytes as evidenced by colabeling of active caspase-3 with TUNEL-positive nuclei. Moreover, our data authenticate with the previously published data in DIC and infarcted hearts (2,4,13,14,22). As per the best of our knowledge, we are the first to report that transplanted CM or ES cells suppress apoptosis in DIC.

Next, to comprehend mechanisms by which CM or ES cells inhibit apoptosis in DIC, we explored the role of PI3K/Akt and ERK pathways. The PI3K/Akt pathway controls regulation of various cellular processes, including cell growth, proliferation, and survival (6,23). In the present study, we imply that DOX treatment significantly decreases levels of Akt. This reduction in Akt was reversed following treatment with CM or ES cells, suggesting a role of Akt in cell survival in DIC hearts. In contrast, the role of ERK pathway is not well defined

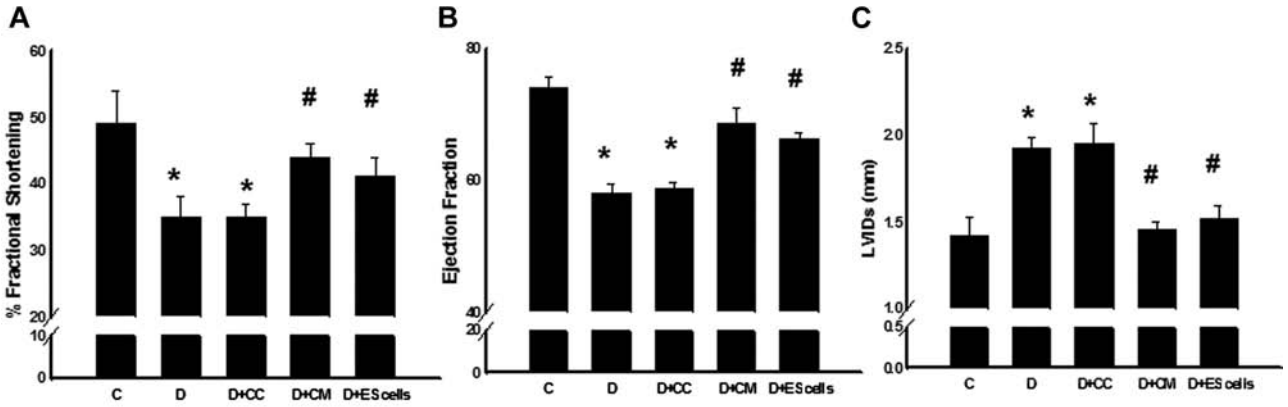

Figure 8. Transplanted CM or ES cells in DIC improve cardiac function at 2 weeks. (A) Average echocardiographic left ventricular fractional shortening. (B) Ejection fraction. (C) Left ventricular interior systolic diameter (LVIDs) for different treatment groups. *$p<0.05$ compared to C; #$p<0.05$ versus D and D+CC.

in assorted heart diseases and considered controversial as it has been shown to be actively involved in cell survival, hypertrophy, and cell death (18,23,32). However, the expression levels of ERK1/2 have been increased in the rat hearts at 4 h post-DOX treatment, whereas this level was decreased at 24 h and 3 weeks posttreatment (19). Our data suggest upregulation of ERK pathway in DOX-treated hearts, suggesting ERK may induce hypertrophy or cell death at 2 weeks in DIC. Interestingly, this increase in ERK pathway was significantly reversed in the hearts treated with CM or ES cells. Varied difference in ERK1/2 in post-DIC can be explained as (1) we used injected DOX 12 mg/kg cumulative dose over the period of 1 week whereas previous report injected 15 mg/kg over the period of 2 weeks (19), which may have generated some variation in the induced cardiomyopathy, which also alters cell signaling. Moreover, authors examined levels of ERK1/2 at 1–24 h and 3 weeks post-DIC (19) compared with our study as we report ERK1/2 levels at 2 weeks post-DIC.

Next, we scrutinized whether transplanted CM or ES cells can inhibit cardiac fibrosis in the post-DIC hearts. Cardiac fibrosis, a major contributor to end-stage heart failure, is the result of heightened levels of various proteinases such as MMPs as well as a decrease in their inhibitors (tissue inhibitor metalloproteinases, TIMPs), which leads to ECM degradation, collagen protein deposition, hardening of the contractile heart muscle, and eventually, cardiac dysfunction (10). We report in the current study that fibrosis considerably exists in DIC hearts. However, presence and amount of fibrosis in DIC compared with infarcted hearts is substantially different (10). Observed fibrosis in the DIC hearts was patchy and widely distributed. In contrast, infarcted hearts exhibit a transmural infarction and significant extent of fibrosis in the confined area (10). The accurate mechanism for the stimulation of fibrosis in the DIC hearts remains obscure, whereas transforming growth factor β1 (TGF-β1) actively induces fibrosis in the infarcted hearts (10). Next, our data for the first time reports that cardiac fibrosis in the DIC hearts was significantly attenuated following CM or ES cell transplantation. We published recently that factors released from ES cells contain antifibrotic factor TIMP-1 and inhibit apoptosis in the H9c2 cells (30,32). We also published that apoptosis and fibrosis in the infarcted hearts was significantly reduced following CM transplantation (31). Moreover, inhibited apoptosis in the H9c2 cells was mediated by Akt pathway but not by ERK pathway (32). However, we suggest that DIC involves both Akt and ERK pathways in the regulation of cardiac remodeling in the present study. Therefore, this raises further questions whether inhibited apoptosis and fibrosis in DIC following CM or ES cell transplantation involves similar mechanisms.

Cytoplasmic vacuolization and myofibrillar loss in cardiac myocytes is well established, and recorded characteristics of DIC were ascertained in animal models and patients (26,27). Our present study also demonstrates the appearance of cytoplasmic vacuolization and myofibrillar loss in DIC. Next, we scrutinized the effects of transplanted CM or ES cells in DIC. We showed that CM or ES cells significantly inhibit cytoplasmic vacuolization and myofibrillar loss in DIC compared with respective controls. Our data are in compliance with the previously published report on the carbon monoxide/heme oxygenase (CO/HO) system that inhibits cytoplasmic vacuolization and myofibrillar loss in DIC (34). In the present study, we confirmed evidence of cytoplasmic vacuolization and myofibrillar loss and extended our findings by proving for the first time that CM or ES cells attenuate cytoplasmic vacuolization and myofibrillar loss in the DIC.

In our present study, we were unable to identify transplanted ES cells in the heart (data not shown); however, there were significant improvement in cardiac function, and therefore, we examined the role of c-kit^{+ve} CSCs. We proposed that autocrine or paracrine factors released from ES cells will activate and differentiate resident CSCs into mature cardiac cell types and regenerate the DOX-injured myocardium. CSCs are undifferentiated, self-renewing, clonogenic, and capable of differentiating into all three major heart cell types both in vitro and in vivo (3). These cells have been identified and proven to regenerate infarcted myocardium (3). In the present study, we demonstrate increased levels of c-kit^{+ve} CSCs following CM or ES cell transplantation compared with their controls. Our data are in accordance with the previously published studies showing c-kit^{+ve} cells in the infarcted heart were up regulated and differentiated into cardiac myocytes following treatment with HGF and IGF-1 growth factors (35). Therefore, we extended our findings and determined whether levels of HGF and IGF-1 are being affected in the DIC with or without CM or ES cell treatment. To our surprise, we observed significant increased levels of HGF and IGF-1 growth factors in CM and ES cell-treated hearts compared with DOX, DOX + CC, and normal saline groups, suggesting these released factors might have played a significant role in their activation. Therefore, further studies are required to determine if more than two growth factors reported in this study are involved in the inhibition of multiple pathological conditions observed in DIC.

Moreover, we determined whether transplantation of CM or ES cells contributes to improved cardiac function in DIC. In the present study, we have demonstrated that 2 weeks post-DIC, mice transplanted with CM or ES cells had significantly improved cardiac function compared with DIC.

In conclusion, this is the first study proposing that CM or ES cells have the competence to inhibit multiple pathological conditions such as cardiac myocyte apoptosis, Akt and ERK pathways, cardiac fibrosis, cytoplasmic vacuolization, and myofibrillar loss as well as upregulation of c-kit^{+ve} CSCs and their differentiation into cardiac myocytes along with improved cardiac function in DIC.

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