Factors Secreted by Mesenchymal Stem Cells and Endothelial Progenitor Cells Have Complementary Effects on Angiogenesis In Vitro

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Stem cell-based therapy for myocardial regeneration has reported several functional improvements that are attributed mostly to the paracrine effects stimulating angiogenesis and cell survival. This study was conducted to comparatively evaluate the potential of factors secreted by mesenchymal stem cells (MSCs) in normoxic and hypoxic conditions to promote tissue repair by sustaining endothelial cell (EC) adhesion and proliferation and conferring protection against apoptosis. To this aim, a conditioned medium (CM) was generated from MSCs after 24-h incubation in a serum-free normal or hypoxic environment. MSCs exhibited resistance to hypoxia, which induced increased secretion of vascular endothelial growth factor (VEGF) and decreased levels of other cytokines, including stromal-derived factor-1 (SDF). The CM derived from normal (nMSC-CM) and hypoxic cells (hypMSC-CM) induced similar protective effects on H9c2 cells in hypoxia. Minor differences were noticed in the potential of normal versus hypoxic CM to promote angiogenesis, which were likely connected to SDF α and VEGF levels: the nMSC-CM was more effective in stimulating EC migration, whereas the hypMSC-CM had an enhanced effect on EC adhesion. However, the factors secreted by MSCs in normoxic or hypoxic conditions supported adhesion, but not proliferation, of ECs in vitro, as revealed by impedance-based dynamic assessments. Surprisingly, factors secreted by other stem/progenitor cells, such as endothelial progenitor cells (EPCs), had complementary effects to the MSC-CM. Thus, the EPC-CM, in either a normal or hypoxic environment, supported EC proliferation, but did not sustain EC adhesion. Combined use of the MSC-CM and EPC-CM promoted both EC adhesion and proliferation, suggesting that the local angiogenesis at the site of ischemic injury might be better stimulated by simultaneous releasing of factors secreted by multiple stem/progenitor cell populations.

Introduction

THE STEM CELL THERAPY for postinfarct myocardial regeneration has been introduced more than 2 decades ago, but thus far, the functional improvements obtained are still limited due to the low survival rate of transplanted cells into the damaged myocardium [1,2]. Consequently, the clinical benefits are only transient and attributed mostly to transplanted cell-associated paracrine effects that stimulate angiogenesis and confer cardioprotection [3]. In this context, the delivery of the paracrine factors secreted by stem cells might be able to provide a more efficient and attractive approach for the myocardial repair process that circumvents problems typically associated with cell delivery.

The capacity to secrete protective biologically active factors designates mesenchymal stem cells (MSCs) among the most suitable tools for paracrine contribution to tissue

regeneration [4]. Other advantages that make MSCs useful for such therapy include their convenient isolation (from either bone marrow or adipose tissue), trophic activity, lack of immunogenicity or ethical controversy [5], as well as their potential to differentiate into specific cell types [6], and promote vascularization [7]. In addition to MSCs, several other cell populations, such as hematopoietic stem cells and endothelial progenitor cells (EPCs), have been shown to augment functional recovery after experimentally induced ischemia [8], and are currently being evaluated in clinical trials for their therapeutic effects [9].

Despite the general consensus on the beneficial effects of adult stem cells and the numerous preclinical and clinical ongoing studies on cell-based therapy for myocardial regeneration, a greater understanding of the mechanisms by which individual stem cell populations confer this protection would be needed to increase the effectiveness of this therapy.

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Equally important, due to the ischemic environment that stem cells face after transplantation into the infarcted myocardium, comprehension of the paracrine properties of stem cells in hypoxic conditions is essential for envisaging appropriate strategies that overcome the potential negative impacts of ischemia. This article investigates the potential of factors secreted by MSCs in normoxic and hypoxic conditions to promote cardioprotection and stimulates chemotraction, engraftment, and proliferation of endothelial cells (ECs). We report here that MSCs and EPCs exert several distinct paracrine effects on ECs that are complementary, and that the combination of the factors they secrete augments the paracrine effects.

Materials and Methods

Cell culture

MSCs with an Sca-1^{pos}/c-kit^{neg}/CD105^{pos}/CD45^{neg}/CD14^{neg}/SMA^{pos} phenotype were isolated from mouse bone marrow as previously described [10]. In compliance to the rules of the International Society for Cellular Therapy, the cells were evaluated for their multipotency, and the results confirmed their capacity to generate adipocytes, osteoblasts, and chondrocytes under appropriate culture conditions. Cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% MSC-qualified fetal bovine serum (FBS; Invitrogen) at 37°C in a humidified incubator containing 5% CO₂ and used between the 8th and 12th passages.

Primary cultures of EPCs were obtained from human peripheral blood of healthy volunteers, with written informed consent for the collection, analysis, storage, and reuse. All patient data were maintained anonymous, and the procedure was approved by the Institutional Ethics Committee of Institute of Cellular Biology and Pathology "Nicolae Simionescu" of the Romanian Academy, in accordance with the most recent version of the Helsinki declaration of World Medical Association (Ethics Principles for Medical Research Involving Human Subjects, October 2008). EPCs were prepared by cultivating the mononuclear cells in EC growth medium MV2 (PromoCell) on 20 µg/mL fibronectin-coated dishes for 7 days. The resulted cells expressed both markers of the monocyte-macrophage lineage (CD14 and CD45) and ECs (KDR, VEGFR1, VE-Cadherin, and CD31), and had the specific pattern of early outgrowth EPC, as previously described [8,11]. Human umbilical vein ECs (EA.hy926 line) were purchased from American Type Culture Collection (ATCC) and cultured in low-glucose DMEM supplemented with 10% FBS (Gibco). The cardiac myocyte cell line H9c2 was obtained from ATCC and grown in culture in high-glucose DMEM as recommended by the manufacturer.

Induction of cell hypoxia and harvesting the conditioned medium

MSCs were plated at 5000 cells/cm² and grown for 4 days, until ~80% confluent. Cells were starved for 24 h in serum-free DMEM and then incubated in a freshly added serum-free medium and exposed to hypoxic conditions (1% O₂). The hypoxic conditions were induced by using an infrared, water-jacked CO₂/O₂ incubator with an increased relative humidity (HERAcell 150). The serum-free medium used for hypoxic treatment was previously made hypoxic by pre-

equilibration in an oxygen-deficit atmosphere (1% O₂, induced by nitrogen replacement) for 24 h, before being added onto the cells. Cells were maintained in a hypoxia-pre-equilibrated serum-free DMEM at 37°C in a constant atmosphere with 5% CO₂ and 1% O₂, for 24 h, and conditioned medium (CM) was generated at the end of this hypoxic treatment (hypMSC-CM). In parallel, control cells were incubated in normoxic conditions and used to generate the nMSC-CM.

Primary cultures of EPCs were washed with phosphate-buffered saline (PBS) and incubated in EC basal medium MV2 for 24 h. Cultured EPCs were further shifted under either normal or hypoxic conditions in hypoxia-pre-equilibrated basal medium MV2 for 24 h, to generate CM from normal (nEPC-CM) and hypoxic EPCs (hypEPC-CM).

After hypoxic treatment, the culture medium was collected and centrifuged at 2500 g, 20 min, 4°C. The supernatant was concentrated 10 times by ultrafiltration using centrifugal filter units with a 10-kDa cut-off (Millipore) and stored in aliquots at -20°C as concentrated CM. For all experiments, the CM was diluted in fresh serum-free culture medium.

Induction of hypoxia and assessment of apoptosis in H9c2 myocytes

H9c2 cells, seeded at 10,000 cells/cm², were grown for 2 days and then incubated in a medium containing 0.5% serum for 24 h before exposure to hypoxic conditions. Hypoxia was induced as described above, by incubating the cells in hypoxia-pre-equilibrated serum-free medium and 1% O₂ atmosphere, in the presence of either the nMSC-CM or the hypMSC-CM. After 24 h of hypoxia, cells were either processed for the evaluation of apoptosis and mitochondrial membrane potential ($\Delta\Psi_m$) or moved to normoxic conditions for real-time monitoring of the cell response to hypoxia reoxygenation. Apoptosis was quantified by Hoechst nuclear staining [12]. To this aim, cells were fixed/permeabilized with ice-cold ethanol and exposed to Hoechst 33258 (0.1 µg/mL PBS) for 10 min at room temperature before examination under the epifluorescence microscope (Nikon Microphot-SA). The apoptotic cells were recognized by their fragmented and condensed nuclei; the dividing cells characterized by condensed DNA (nuclei in metaphases) were excluded from counting.

Measurement of $\Delta\Psi_m$

$\Delta\Psi_m$ was assessed using the lipophilic cationic probe JC-1, which exhibits potential-dependent accumulation in mitochondria. After hypoxic treatment of the cells, the medium was removed, and cells were incubated in DMEM containing 5 µg/mL JC-1 for 10 min at room temperature in the dark. After washing with Hank's balanced saline solution (HBSS), the cells were detached with the rubber policeman and resuspended by centrifugation (400 g, 5 min, 25°C) in 500 µL HBSS, and the fluorescence was monitored using a TECAN iGenios spectrofluorimeter. The collapse of $\Delta\Psi_m$ was examined for each sample and expressed as the ratio between 535 nm and 610 nm fluorescence. The method is based on the ability of JC-1 to form aggregates (denoting accumulation within the mitochondria) leading to high

values at 610 nm fluorescence and indicating normal mitochondrial membrane potential. Loss of $\Delta\Psi_m$ generates reduction of 610-nm fluorescence and a concomitant increase at 535-nm fluorescence (monomeric state of JC-1).

Assays for apoptosis and cell survival

Apoptosis was assessed in cells incubated under normal and hypoxic conditions by Caspase-3 activity and real-time polymerase chain reaction (PCR) estimation of the mRNA ratio between the pro- and antiapoptotic proteins of the Bcl-2 family. Caspase-3 activity was determined using a fluorescent kit from R&D Systems following the protocol recommended by the manufacturer. Cell proliferation was assessed by MTT assay, as previously described [12].

The effect of CM on the adhesion and proliferation of ECs was evaluated with the xCELLigence System (Roche Applied Science). This system monitors cellular events in real time by measuring electrical impedance across interdigitated microelectrodes integrated on the bottom of specially designed tissue culture E-plates. The effect of CM on cell adhesion was evaluated by plating ECs onto E-plates in the presence of the nMSC-CM and hypMSC-CM and tracking the dynamics of cell attachment and proliferation. To investigate the effects of the MSC-CM on EC proliferation, separate experiments were performed in which the CM was added onto cell culture after ECs adhered onto substrate. Briefly, ECs were cultured on E-plates in a serum-containing DMEM, and 3 h later, once all cells had adhered to the substrate, the medium was replaced with a serum-free DMEM containing the CM. Controls of ECs incubated in no serum- and serum-containing medium were included.

Chemotaxis assay

CIM plates were employed equipped with an intercalated membrane with 8- μ m pores and microelectrodes onto their inner face generating impedance signal when come in contact with migrated cells. EC suspension (400,000 cells/mL) was added onto the upper well of the CIM plate (100 μ L/well); the lower well contained the culture medium supplemented with the CM. Wells containing no serum and 10% serum were included as negative and positive controls, respectively. The migration of the cells onto the inner face of the upper wells in response to the nMSC-CM and the hypMSC-CM was dynamically monitored by increasing the electrical impedance over 3 h.

ELISA assay

The levels of vascular endothelial growth factor (VEGF), stromal-derived factor-1 (SDF) and transforming growth factor- β (TGF- β) secreted by nMSC and hypMSC were quantified by ELISA assay (R&D Systems) in an MSC supernatant (not concentrated CM) and cell lysate, and the data were normalized to the total protein. The active secretion of the pro- and active forms of MMP-9 by MSCs was monitored using ELISpot (R&D Systems). Positive and negative controls provided by the manufacturer were performed in parallel for comparisons.

Cytokine array

The profile of the relative levels of multiple cytokines in the MSC supernatant was analyzed using Mouse Cytokine

Array Panel A (R&D Systems). Briefly, the supernatant derived from normal and hypoxic MSCs was mixed with the cocktail of biotinylated detection antibodies (provided by the manufacturer) and then incubated with the nitrocellulose (NC) membrane containing each of the capture antibodies spotted in duplicate. After washing to remove unbound material, the membrane was incubated with Streptavidin-HRP and then with chemiluminescent detection reagents (Thermo Scientific). The light produced at each spot was detected with FUJIFILM Luminescent Image Analyzer LAS-3000, and pixel densities were analyzed with TotalLab Quant software.

Matrigel plug assay

The mice were housed and used in accordance with the Guide for the Care and Use of Laboratory Animals (Washington DC, National Academies Press, 1996), and all experiments were approved by the Institutional Ethics Committee of Institute of Cellular Biology and Pathology "Nicolae Simionescu" of the Romanian Academy. The mixtures of 450 μ L of growth factor-reduced Matrigel (BD Biosciences) and 50 μ L of nMSC-CM or hypMSC-CM or 300 ng FGFb were prepared on ice and implanted into 10-week-old male C57Bl/6 mice under ketamine-xylazine-acepromazine anesthesia (80/20/1, i.p.) by subcutaneous injection. The Matrigel, needle, and syringe were kept on ice until the time of injection to prevent gelling. For each experimental condition, 3 mice, each one with 2 implantations in the dorsal surfaces at the paraspinal space using 25-gauge needles, were employed. On day 7, Matrigel plugs were surgically excised from the reanesthetized mice and processed for paraffin embedding and hematoxylin-eosin staining to evaluate the infiltrating cells.

Scratch test assay

ECs were grown to confluence on 24-well plates. Cells were scratched using a 200- μ L tip and immediately washed and incubated in the presence of MSC-CM. The cells were photographed immediately after addition of CM ($t=0$ h) and 8 h later ($t=8$ h). Cell spreading over the scratched area was assessed as a function of the scratched area covered with cells after 8 h. The covered area was estimated in square pixels using the AxioVision Rel.4.8 program (Carl Zeiss Vision).

Real-time PCR

Total RNA was extracted using the RNeasy Micro kit (Qiagen), and cDNA was synthesized from 0.2 μ g of total RNA by using a mix of oligo(dT) and random primers with MMLV reverse transcriptase (Invitrogen). Real-time PCR was performed following the optimization of the amplification conditions for each set of primers. The comparative C_T method was used to quantify the results, and 18S was used for internal normalization [13].

Western blot assay

After exposure to normoxic or hypoxic conditions, MSCs were washed with PBS and scraped off in hot Laemmli's loading buffer. Equal amounts of protein extracts (20 μ g/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel

electrophoresis on a 5% stacking gel and a 10% separating gel, and then proteins were transferred onto NC membrane (20 min at 10 V). After blocking in PBS containing 0.05% Triton X-100 (TBS) and 5% milk for 1 h, the NC membranes were incubated overnight at 4°C with anti-TGF- β 1 or anti- β -actin primary antibodies. Washed membranes were incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG) for 1 h. Antigen-antibody complexes were visualized by chemiluminescence with an ECL kit (Thermo Scientific).

Statistics

All data are expressed as the mean $\pm$ S.D from at least 3 experiments. Statistical analysis was performed by one-way ANOVA test. When this analysis indicated significance, Bonferroni's multiple comparison test from GraphPad Prism 5 software. A P -value < 0.05 was considered significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$).

Results

Resistance of MSCs to hypoxia-induced apoptosis

The direct response of cells to hypoxic conditions was confirmed by the increase of heme oxygenase-1 protein level (Fig. 1a). To test whether hypoxia induces MSC death, the hypoxic cells were evaluated for Caspase-3 activity and expression of pro- and antiapoptotic genes. The viability of the

cells after incubation under hypoxia was 90% of the normal cells (data not shown). Hypoxia induced 1.7-fold increase in Caspase-3 activity in MSCs (Fig. 1b), but did not significantly modified the *bax/bcl-2* mRNA ratio (Fig. 1c). These 2 members of the Bcl-2 family of proteins, Bax and Bcl-2, have essential yet opposite roles in regulating apoptosis, and their ratio is a useful estimation of apoptosis being indicative of changes in mitochondrial permeability and ensuing release of cytochrome c. The resistance of MSCs to hypoxic conditions was further supported by the experiments in which $\Delta\Psi_m$ was determined. The $\Delta\Psi_m$ of MSCs was not modified by hypoxic conditions, as revealed by presence of JC-1 red aggregates (610 nm) and no JC-1 green monomer (535 nm) within the cells, indicating JC-1 accumulation within normal mitochondria by the end of the hypoxia treatment (Fig. 1d). These results, which are indicative of a relative resistance of MSCs to hypoxia, corroborate well with the previously reported resistance and preservation of the differentiation potential of MSCs in ischemia [14].

Comparative analysis of angiogenic factors secreted by MSCs in normal and hypoxic conditions

Comparative analysis of the angiogenic factors in the cell supernatant of normal and hypoxic MSCs revealed that the VEGF level secreted by hypoxic MSCs was significantly increased as compared to the level secreted by nMSCs (363 ± 83 pg VEGF/mg protein in hypoxia vs. 220 ± 46 pg VEGF/mg protein in normoxia) (Fig. 2a). However, no significant difference was noticed in the expression of TGF- β in MSCs (at either mRNA or protein levels) between normoxic and hypoxic conditions (Fig. 2b), nor in the secreted level in the culture supernatant (270 ± 17 pg TGF- β 1/mg protein in hypoxia vs. $296 \pm s$ pg TGF- β 1/mg protein in normoxia). On contrary, a decreased amount of SDF was found at both mRNA levels (Fig. 2c), as well as by ELISA, in the MSC lysate and culture supernatant (Fig. 2d).

As the efficacy of stem cells in cell transplantation therapy relies on their ability to preserve the active secretion of protective factors, the comparative profile of the secreted cytokines was analyzed in MSC supernatant in normal and hypoxic conditions. The results revealed that MSCs secreted high levels of proangiogenic cytokines, that is, SDF α , CXCL-1, RANTES, MCP-1, and M-CSF (Fig. 2e). Several antiangiogenic cytokines, namely TIMP-1, IL-1ra, and CXCL-10/IP-10, were also identified in a large amount (Fig. 2e). Unless TIMP-1 (whose secreted levels remained similar) and CXCL-10/IP-10 (which almost completely disappeared in MSC supernatant during exposure to hypoxia), all these pro- and anti-angiogenic cytokines were still secreted under hypoxic conditions, although their secreted level was lower in hypoxic versus normoxic conditions (Fig. 2f). Furthermore, after the hypoxic exposure, MSCs still secreted high levels of pro- and active forms of MMP-9 (Fig. 2g). These results showed that MSCs partially preserved their innate properties in hypoxic conditions.

Protective effects of factors secreted by normal and hypoxic MSCs on myocytes

Incubation of H9c2 cells in hypoxic conditions for 24 h induced a loss of the $\Delta\Psi_m$, as demonstrated by the

FIG. 1. The effect of hypoxia on mesenchymal stem cells (MSCs). **(a)** Increase of heme oxygenase-1 (HMOX-1) protein level in MSCs after 24-h exposure to hypoxic conditions. lane 1: normal MSCs (before starvation); lane 2: normoxic MSCs (after 24-h incubation in serum-free DMEM and hypoxic conditions); lane 3: hypoxic MSCs. **(b)** Minimal increase in Caspase-3 activity (1.7-fold as compared to normal cells) was determined in MSCs after hypoxic treatment. The data represent the mean values from 5 independent experiments performed in duplicate (* $P < 0.05$). **(c)** The *bax/bcl-2* mRNA ratio in MSCs after exposure to hypoxia, as determined by RT-polymerase chain reaction (PCR) analysis. The data represent the mean values from 3 independent experiments performed in triplicate. **(d)** Fluorescence microscopy of MSC after hypoxic treatment illustrating the accumulation of JC-1 within mitochondria as red aggregate (demonstrating normal mitochondrial membrane potential [$\Delta\Psi_m$] and intact mitochondria) and no evidence of JC-1 green monomers into the cytosol. DMEM, Dulbecco's modified Eagle's medium; RT, real time.

FIG. 2. Angiogenic factors secreted by MSCs in normal and hypoxic conditions. **(a)** Vascular endothelial growth factor (VEGF) level quantified in the culture supernatant after 24-h incubation of MSCs in normal and hypoxic conditions (ELISA assay). The data represent the mean values from 5 independent experiments (* $P < 0.05$). **(b)** Stable expression (at both protein and mRNA levels) and constant secretion levels of transforming growth factor- β 1 (TGF- β 1) in MSCs after exposure to hypoxia. **(c)** ELISA quantification of SDF-1 in the MSC lysate and supernatant in normal and hypoxic conditions. The data represent the mean values from 3 (cell lysate) and 5 (supernatant) independent experiments (*** $P < 0.005$). **(d)** Real-time PCR quantification of SDF-1 expression in normal and hypoxic MSCs. The data represent the mean values from 3 independent experiments performed in triplicate. **(e)** The profile of MSC-secreted cytokines in normal conditions. The image is representative of 3 experiments. **(f)** Quantification of the cytokine expression level in MSC supernatant obtained in normal and hypoxic conditions. The data represent the mean values of 3 experiments performed in duplicates. **(g)** Qualitative ELISpot determination of the pro- and active forms of MMP-9 released from MSCs after exposure to hypoxic conditions.

appearance of diffuse green monomer fluorescence detected after cell incubation with JC-1 (Fig. 3a, upper pictures). The JC-1 fluorescence was quantified by spectrofluorimetry, and the $\Delta\Psi_m$ loss was confirmed by the increase in the 535 nm/610 nm fluorescence ratio (Fig. 3a, diagram). The presence of nMSC-CM or hypMSC-CM in the culture medium of myocytes during exposure to hypoxic conditions resulted in the attenuation of $\Delta\Psi_m$ loss as compared to cells incubated in hypoxic conditions in a serum-free medium. These results imply the presence of mitochondrial preserving factors in both the nMSC-CM and hypMSC-CM.

H9c2 apoptosis induced by the hypoxic treatment was quantified by DNA Hoechst staining (Fig. 3b) and Caspase-3 activity assay (Fig. 3c). The results revealed that both the percentage of apoptotic nuclei (Fig. 3b) and Caspase-3 activation (Fig. 3c) induced by H9c2 exposure to hypoxia and no-serum medium were significantly reduced in the presence

of the MSC-CM. Notably, similar protective effects were observed for the nMSC-CM and hypMSC-CM on myocytes. The data suggest that MSCs, in either normal or hypoxic conditions, secrete paracrine factors with a cardioprotective role.

To validate these results, the dynamic response of myocytes to hypoxia treatment followed by reoxygenation was assessed by the xCELLigence system that monitors the cellular events in real-time by measuring the electrical impedance across interdigitated microelectrodes integrated on the bottom of tissue culture E-Plates. Cells were seeded onto E-Plates for 2 days before being incubated in DMEM containing 0.5% serum for 24 h (starvation), and then exposed to hypoxic conditions in the presence of nMSC-CM or hypMSC-CM (Fig. 2d). Hypoxia reoxygenation in a no-serum condition resulted in H9c2 cell death as revealed by continuous decrease of cell index values after hypoxia initiation. The

FIG. 3. Protective effects of MSC-conditioned medium (CM) on cardiac myocytes in hypoxic conditions. **(a)** (Top) Representative picture of myocytes stained with JC-1 after exposure to hypoxia. Note that polarized mitochondria appear as punctuate red fluorescence, whereas depolarized mitochondria exhibit the characteristic diffuse monomer fluorescence. (Below) Assessment of $\Delta\Psi_m$ loss expressed as the increase in 535 nm/610 nm fluorescence ratio and the effect of normal MSC-CM (nMSC-CM) and hypoxic MSC-CM (hypMSC-CM) in preventing $\Delta\Psi_m$ loss (left). Data are mean $\pm$ S.D. of results obtained in 1 experiment and representative of 3 ($***P < 0.005$). **(b)** Quantitative determination of apoptotic H9c2 nuclei after exposure to hypoxia in the presence and absence of MSC-CM. The diagram represents the percentage of apoptotic cell nuclei determined by Hoechst staining. A minimum of 1,000 cells per assay was counted. The upper panel shows the fluorescence microscopy of Hoechst staining reaction illustrating apoptotic cells (right, arrowheads) and several dividing cells (left, arrows) that were excluded from the counting ($*P < 0.05$). **(c)** The level of Caspase-3 activity determined in H9c2 cells after 24-h exposure to hypoxia and the protective role of nMSC-CM and hypMSC-CM on the Caspase-3 activation. The data represent the mean values from 5 independent experiments performed in duplicate. **(d)** Dynamic assessment of myocyte response to hypoxia and reoxygenation in the presence of MSC-CM (original recording). Cells incubated in culture medium with no serum define the baseline, and the plot shows data normalized to the time point before the beginning of starvation (1 representative experiment from 3). The right diagram illustrates the normalized cell index values at 24 h after reoxygenation. The average is resulted from 3 independent experiments performed in duplicates ($**P < 0.01$). Color images available online at www.liebertpub.com/scd

presence of either nMSC-CM or hypMSC-CM induced higher cell index values starting from 12 h of hypoxia (time point 90), and these values maintained increased in comparison to no serum even after 24 h of reperfusion (Fig. 3d, diagram). Nevertheless, in comparison to serum, the nMSC-CM and hypMSC-CM had much lesser effects, suggesting a protective role of CM on cell survival only, but no effect on cell proliferation after reperfusion. Altogether, these data demonstrated that the MSC-CM conferred protection on myocytes against hypoxia-induced apoptosis, and these protective effects were similar in normal and hypoxic conditions.

Chemoattractant properties of factors secreted by MSCs in normal and hypoxic conditions

To test whether the MSC-CM has chemoattractant properties *in vivo*, the effect of the nMSC-CM and hypMSC-CM was comparatively assessed by Matrigel plug assay. Growth

factor-reduced Matrigel, alone and mixed with FGfb, was used as negative and positive control, respectively. Histological examinations of plugs revealed a massive cellular invasion of the Matrigel in the presence of FGfb. In comparison to Matrigel alone, the presence of nMSC-CM or hypMSC-CM induced the colonization of the implanted Matrigel with inflammatory cells and the formation of capillary-like structures (Fig. 4a, arrowheads). Quantification of the cells invaded into the Matrigel revealed no significant difference in the chemotactic effect of nMSC-CM and hypMSC-CM (Fig. 4a, diagram). These observations are consistent with the idea that the MSC-CM contains factors that induce EC invasion and neovessel formation *in vivo*, irrespective of the hypoxic or normoxic conditions.

To test whether factors secreted by normal and hypoxic MSCs have the potential to induce EC migration, CIM Plates and xCELLigence System were employed. Migration of ECs across the microporous membrane evaluated as a function of time showed that like the serum but to a lesser extent, both

FIG. 4. The vasculogenic potential of MSC-CM. **(a)** Matrigel containing nMSC-CM, hypMSC-CM, or FGFb evaluated after 7 days from in vivo implantation. *Arrowheads* illustrate capillary-like structures. The diagram illustrates the quantification of the cells within the Matrigel ($*P < 0.05$; $**P < 0.01$). **(b)** Original recording illustrating the time-dependent chemotactic migration of EA.hy926 (in duplicates) in response to nMSC-CM and hypMSC-CM. The diagram on the *right* illustrates the cell migration index of endothelial cells (ECs) after 3 h. The average is resulted from 3 independent experiments performed in duplicates. **(c)** Scratch test assay on ECs. (*left*) Cells were scratched, photographed at time 0, and incubated in the presence of nMSC-CM and hypMSC-CM. Photographs were taken again after 8 h of culture. (*right*) Quantification of the covered area as percentage of the initial scratched area. The diagram illustrates the mean $\pm$ S.D. of 3 independent experiments ($***P < 0.005$). Color images available online at www.liebertpub.com/scd

the nMSC-CM and hypMSC-CM were chemoattractant for ECs (Fig. 4b, left side). The maximum chemoattractant effect of MSC-CM was achieved 2 h after seeding and subsequently plateaued. Although both the nMSC-CM and hypMSC-CM stimulated EC migration in vitro, the chemotactic activity of the hypMSC-CM was lower compared to the nMSC-CM. Quantification of cell migration index after 3 h (Fig. 4b, right side), when the chemotactic effect produced by serum appeared highest, showed that the effect produced by the nMSC-CM and the hypMSC-CM was around half of that produced by the same amount of serum. This effect is quite significant, considering that the CM was collected from cells after 24 h of culture. The migratory effect of the MSC-CM on ECs was also analyzed by a scratch test assay. As shown in Fig. 4c, the nMSC-CM and the hypMSC-CM had consistent and similar paracrine contributions to EC spreading, and produced effects comparable to serum.

Taken together, these data indicated that the MSC-CM has essential paracrine properties on chemotaxis and migration of ECs either in hypoxic or normal conditions, which may be

advantageous for the induction of angiogenesis after transplantation into the infarcted myocardium.

Effects of factors secreted by normal and hypoxic MSCs on EC proliferation

The above results demonstrated the migratory effects of the MSC-CM on ECs. Nevertheless, for the angiogenesis process to be completed, the migration of ECs has to be accompanied by the engraftment and proliferation of cells at the site of infarction. Therefore, the ability of MSC-secreted factors to support EC adhesion and proliferation was dynamically evaluated with the xCELLigence system. To this aim, ECs were plated onto 16-well E-Plates (5000 cells/well) in a DMEM containing the nMSC-CM or the hypMSC-CM, and the effect was evaluated in comparison to cells plated in the absence or presence of serum (negative and positive controls, respectively). Although both the CM have strong effects in inducing the attachment of ECs to the culture substrate, which were comparable to serum, an apparently

FIG. 5. The effect of nMSC-CM and hypMSC-CM on the adhesion and proliferation of ECs in vitro (original recordings). **(a)** Dynamic assessment illustrating the initial EC attachment to E-plate surface under the influence of nMSC-CM and hypMSC-CM as compared to serum or no-serum controls. The diagram below illustrates the mean values of cell indexes at 3 h after plating (resulted from 3 experiments) (* $P < 0.05$; ** $P < 0.01$). **(b)** Real-time monitoring of the effect of MSC-CM on the EC adherence and proliferation. The plot shows the ability of nMSC-CM and hypMSC-CM to support EC adhesion and their failure to support EC proliferation in comparison to serum. The diagram below illustrates the cell index 48 h after cell seeding (resulted from 3 experiments) (** $P < 0.005$). **(c)** Real-time monitoring of the effect of nMSC-CM and hypMSC-CM on EC proliferation after adherence. The plot shows data normalized to the last time point before replacing the serum-containing medium with MSC-CM. The diagram below illustrates the normalized cell index 12 h after cell plating (resulted from 3 experiments). All the recordings show the mean values obtained from duplicates and are representatives from 3 independent experiments. Color images available online at www.liebertpub.com/scd

slightly enhanced effect (although with not statistical significance) of the hypMSC-CM was noticed as compared to the nMSC-CM (Fig. 5a). These data suggested that MSC secreted additional factors in hypoxic conditions that could better promote EC adhesion. However, neither hypMSC-CM nor nMSC-CM did properly sustain EC proliferation after

adherence. Thus, starting to 12 h after seeding, ECs were not able to proliferate in the presence of MSC-CM, as they did in the presence of serum (Fig. 5b). In additional experiments, ECs were first allowed to adhere to E-plates in the presence of 10% serum, and subsequently, the culture medium was replaced with DMEM containing nMSC-CM or hypMSC-

FIG. 6. Comparative effects of MSC-CM and endothelial progenitor cell (EPC)-CM on the adherence and proliferation of ECs. **(a)** Effect of MSC-CM and EPC-CM on EC adherence and proliferation (original recording). Note that MSC-CM, but not EPC-CM, sustains EC adherence; however, MSC-CM is not able to support cell proliferation. **(b)** Effect of MSC-CM and EPC-CM on EC proliferation after serum-induced adherence (original recording). In contrast to MSC-CM, EPC-CM supports EC proliferation, at a level comparable to serum. **(c)** Dynamic growth curves of ECs exposed to the culture medium containing both MSC-CM and EPC-CM (original recording). The 3 recordings illustrate representative experiments performed in duplicate, and similar patterns were obtained in 3 experiments with different conditioned media. **(d)** Diagram illustrating EC proliferation (MTT assay) in the presence of MSC-CM, EPC-CM, and the combination of MSC-CM and EPC-CM (** $P < 0.05$; *** $P < 0.005$). Color images available online at www.liebertpub.com/scd

CM. The results showed that the MSC-CM (derived from either normal or hypoxic MSCs) failed to support EC proliferation (Fig. 5c). Quantification of the cell index 12 h after seeding (meaning 3 h of incubation in the presence of serum plus 9 h in the presence of CM) revealed no proliferation effect of nMSC-CM and hypMSC-CM on ECs, which was similar to the negative control (no serum). These data suggested that MSCs (in either normal or hypoxic conditions) did not secrete factors to support proliferation of ECs, and therefore although the CM was able to support EC adherence, it was lacking the ability to promote EC proliferation. These data might explain the inability of MSC to stimulate host cardiac repair solely by paracrine mechanisms, as recently reported [15].

EPCs and MSCs have opposite effects on adhesion and proliferation of ECs

The above results prompted us to evaluate the ability of other progenitor cell populations to promote EC adhesion and proliferation and cardiomyocytes (CMC) protection against apoptosis. The EPC-CM derived from both normal and hypoxic EPC provided similar protective effects on H9c2 apoptosis in hypoxic conditions as MSC-CM (data not shown). Conversely, when EA.hy926 cells were plated in E-Plates in the presence of EPC-CM (in similar experimental conditions as described above for cells cultured in the presence of MSC-CM), no increase in impedance was noticed, and the recording was similar to the no-serum condition (Fig. 6a). These results suggested that in contrast to the MSC-CM, the EPC-CM contained no factors to support EC adhesion. Nevertheless, when cells were first allowed to adhere to E-Plates in serum-containing medium and then shifted to the DMEM containing EPC-CM, the dynamic growth curve of the cells was very similar to that of cells grown in 10% serum (Fig. 6b). These data demonstrate the existence of distinct paracrine effects of MSCs and EPCs on EC adhesion and proliferation in vitro.

The above results led us to examine the effect of the combined use of the MSC-CM and EPC-CM on EC adhesion and proliferation. As revealed in Fig. 6c, ECs cultured in a medium simultaneously containing the MSC-CM and EPC-CM displayed both adhesion and cell proliferation, as demonstrated by the continuous increase of the impedance values over time. The results obtained with the xCELLigence system were further confirmed by MTT assay. As revealed in Fig. 6d, EC proliferation was significantly higher at 3 days after seeding in the medium containing the combination of MSC-CM and EPC-CM than in the presence of either individual one.

Taken together, these data demonstrate that factors secreted by MSCs are unable to simultaneously sustain the adhesion and proliferation of ECs. MSCs have several distinct paracrine effects than EPCs, but these effects are complementary, and thus the combination of these 2 cell populations may produce better paracrine effects after transplantation.

Discussions

This study investigated the behavior and paracrine effects of MSCs and EPCs under hypoxic conditions and their

potential to sustain EC adhesion and proliferation, both alone and in combination. The main findings of this work are as follows:

- (i) The secretory activity of MSCs is slightly modified under hypoxic conditions, but these changes have no major impact on MSC paracrine effects.
- (ii) The factors secreted by normal and hypoxic MSCs have similar beneficial paracrine properties on ischemic cardiomyocytes in vitro.
- (iii) In either normal or hypoxic conditions, MSCs secrete factors that stimulate the chemotaxis and adhesion of ECs; however, they are unable to sustain EC proliferation after adhesion.
- (iv) EPCs show complementary effects in vitro to those induced by MSCs; they stimulate EC proliferation, but not EC adhesion.
- (v) Simultaneous adhesion and proliferation of ECs can be successfully achieved by combining CM from MSCs and EPCs.

The adult stem/progenitor cells from various sources have been experimentally shown to augment functional myocardial recovery after infarction because of their ability to release paracrine factors [16–19]. Clinical trials confirmed these data, showing that cell therapy with autologous cells was safe and provided beneficial effects on myocardial regeneration after infarction [9]. However, despite multiple positive preclinical and clinical studies using cell-based therapies for myocardial infarction, an understanding of the mechanisms underlying these beneficial effects is still lacking. Previous studies have reported that the hypoxic environment induced an increase in the percentage of hematopoietic stem cells with long-term engraftment potential, through mechanisms mediated in part by HIF-1 α [20]. These findings were in accordance with the hypothesis that stem cells with long-term engraftment capabilities were predominantly located at the lowest end of an oxygen gradient in the bone marrow, in hypoxic regions containing sinusoids rather than capillary structures [21]. Other studies have shown that stem cells were resistant to hypoxia-induced apoptosis [14], and hypoxia induced the production and secretion of factors that stimulate inflammation and neovascularization [22,23]. The present study confirmed the hypoxia-resistance of MSCs and showed the partial preservation of their innate properties in hypoxic versus normoxic cells. However, the differences in the paracrine secretions noticed between normoxic and hypoxic conditions had no major impact on the protection provided to myocytes and ECs in vitro. The unexpected decreased level of SDF α in hypoxic MSCs, documented not only at the mRNA level but also by ELISA and cytokine array, might explain the lesser chemotactic properties of the hypMSC-CM, as compared to the nMSC-CM, on ECs (Fig. 4b). On the contrary, the increased secreted level of VEGF by hypoxic MSCs might explain the slightly increased effect (not statistically significant) of hypMSC-CM versus nMSC-CM on EC adherence (Fig. 5a).

Even still controversial, numerous reports have recently shown that stem cell transplantation therapy was minimally effective at replacing dead cardiac cells with new contractile cardiomyocytes [16]. Therefore, it is generally believed that the major benefits derived from transplanted stem cells are the paracrine effects of secreted molecules [17]. This

hypothesis was confirmed by numerous studies showing that the CM, collected from a variety of stem cell populations, had beneficial effects on cells and tissues in vitro and in vivo [3,24]. These studies showed that various stem/progenitor cell populations, such as MSCs or EPCs, released proangiogenic and antiapoptotic cytokines in culture, which may contribute to enhanced angiogenesis and endogenous repair [25]. However, for the stem cell-secreted factors to be useful in cell therapy for efficient vascular repair, more research is needed to identify the mechanisms by which they affect vasculogenesis. Broadly speaking, vascular regeneration includes the restoration of normal vascular function and structure and the growth of new blood vessels [26]. In terms of in vitro studies, these mean increased chemoattraction, engraftment, survival, and proliferation of ECs.

This study provides insights into the functional differences between 2 stem cell populations that likely reflect different signaling pathways by which the soluble factors can effectively substitute the effects of stem cells after transplantation. The inability of MSC to stimulate host cardiac repair solely by paracrine mechanisms was also recently noticed by in vivo studies, on experimentally induced myocardial infarction in pigs [15]. Hatzistergos et al. demonstrated that transplanted MSCs, but not MSC-CM, engrafted and responded directly to cues of injury in the infarcted myocardium by stimulating cardiac progenitor cells to proliferate and differentiate [15]. Our study corroborates the previous reported study, showing that the MSC-CM is not able to support effective angiogenesis by its own, but this might be achieved by combining the MSC-CM with EPC-CM.

MSC transplantation into the myocardium was shown to promote effective angiogenesis by inducing mitotic activity in ECs, in addition to chemoattraction and engraftment. The complete angiogenesis observed after stem cell transplantation into the infarcted myocardium may be explained by the different behavior of stem cells in culture and by the different secretion activity of cells mediated by in vivo cell-cell interactions. The new findings of our study are apparent when considering the replacement of stem cell transplantation therapy with the delivery of the progenitor cell-secreted factors into the infarcted myocardium as an alternative cost-saving solution with similar benefits. If such a therapeutic option is to be considered, the identification of the cocktail of components secreted by the stem cell populations is needed, but not sufficient. The studies must confirm the ability of the cocktail to support chemoattraction, engraftment, survival, and proliferation of ECs, in addition to the beneficial effects on cardiomyocyte protection.

In conclusion, our data demonstrate that MSCs and EPCs exert several distinct paracrine effects on ECs and suggest that neither one by itself may simultaneously support both adhesion and proliferation of ECs. Since their effects are complementary, the combination of these 2 progenitor cell populations or the factors they secrete have a profound positive effect on EC behavior in vitro and may successfully stimulate the angiogenesis in vivo, after transplantation.

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Author Disclosure Statement

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References

1. Wei H, TH Ooi, G Tan, SY Lim, L Qian, P Wong and W Shim. (2010). Cell delivery and tracking in post-myocardial infarction cardiac stem cell therapy: an introduction for clinical researchers. *Heart Fail Rev* 15:1–14.
2. Kang S, YJ Yang, CJ Li and RL Gao. (2008). Effects of intracoronary autologous bone marrow cells on left ventricular function in acute myocardial infarction: a systematic review and meta-analysis for randomized controlled trials. *Coron Artery Dis* 19:327–335.
3. Baraniak PR and TC McDevitt. (2010). Stem cell paracrine actions and tissue regeneration. *Regen Med* 5:121–143.
4. Kinnaird T, E Stabile, MS Burnett, CW Lee, S Barr, S Fuchs and SE Epstein. (2004). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 94:678–685.
5. Zhang M, N Mal, M Kiedrowski, M Chacko, AT Askari, ZB Popovic, ON Koc and MS Penn. (2007). SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J* 21:3197–3207.
6. Sasaki M, R Abe, Y Fujita, S Ando, Inokuma and H Shimizu. (2008). Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol* 180:2581–2587.
7. Martens TP, F See, MD Schuster, HP Sondermeijer, MM Hefti, A Zannettino, S Gronthos, T Seki and S Itescu. (2006). Mesenchymal lineage precursor cells induce vascular network formation in ischemic myocardium. *Nat Clin Pract Cardiovasc Med* 3:S18–S22.
8. Sieveking DP, A Buckle, DS Celermajer and MKC Ng. (2008). Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. *J Am Coll Cardiol* 51:660–668.
9. Lipinski MJ, GG Biondi-Zoccai, A Abbate, R Khianey, I Sheiban, J Bartunek, M Vanderheyden, HS Kim, HJ Kang, BE Strauer and GW Vetrovec. (2007). Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. *J Am Coll Cardiol* 50:1761–1767.
10. Rosca A-M and A Burlacu. (2011). Effect of 5-azacytidine: evidence for alteration of the multipotent ability of mesenchymal stem cells. *Stem Cells Dev* 20:1213–1221.
11. Hirschi KK, DA Ingram and MC Yoder. (2008). Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 28:1584–1595.
12. Burlacu A, V Jinga, AV Gafencu and M Simionescu. (2001). Severity of oxidative stress generates different mechanisms of endothelial cell death. *Cell Tissue Res* 306:409–416.

13. Pfaffl MW. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acid Res* 29:2003–2007.
14. Mylotte LA, AM Duffy, M Murphy, T O'Brien, A Samali, F Barry and E Szegezdi. (2008). Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. *Stem Cells* 26:1325–1336.
15. Hatzistergos KE, H Quevedo, BN Oskoue, Q Hu, GS Feigenbaum, IS Margitich, R Mazhari, AJ Boyle, JP Zambrano, et al. (2010). Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ Res* 107:913–922.
16. Collins JM and B Russell. (2009). Stem cell therapy for cardiac repair. *J Cardiovasc Nurs* 24:93–97.
17. Maltais S, JP Tremblay, LP Perrault and HQ Ly. (2010). The paracrine effect: pivotal mechanism in cell-based cardiac repair. *J Cardiovasc Transl Res* 3:652–662.
18. Dimmeler S and D Losordo. (2011). Stem cells review series: an introduction. *Circ Res* 109:907–909.
19. Potapova IA, GR Gaudette, PR Brink, RB Robinson, MR Rosen, IS Cohen and SV Doronin. (2007). Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem Cells* 25:1761–1768.
20. Eliasson P, M Rehn, P Hammar, P Larsson, O Sirenko, LA Flippin, J Cammenga and JI Jönsson. (2010). Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. *Exp Hematol* 38:301–310.
21. Kubota Y, K Takubo and T Suda. (2008). Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochem Biophys Res Commun* 366:335–339.
22. Wyler von Ballmoos M, Z Yang, J Völzmann, I Baumgartner, C Kalka and S Di Santo. (2010). Endothelial progenitor cells induce a phenotype shift in differentiated endothelial cells towards PDGF/PDGFR β axis-mediated angiogenesis. *PLoS One* 5:e14107–e14116.
23. Prado-Lopez S, A Conesa, A Armiñán, M Martínez-Losa, C Escobedo-Lucea, C Gandia, S Tarazona, D Melguizo, D Blesa, et al. (2010). Hypoxia promotes efficient differentiation of human embryonic stem cells to functional endothelium. *Stem Cells* 28:407–418.
24. Blasi A, C Martino, L Balducci, M Saldarelli, A Soleti, SE Navone, L Canzi, S Cristini, G Invernici, EA Parati and G Alessandri. (2011). Dermal fibroblasts display similar phenotypic and differentiation capacity to fat-derived mesenchymal stem cells, but differ in anti-inflammatory and angiogenic potential. *Vasc Cell* 3:5–18.
25. Ruvinov E, T Harel-Adar and S Cohen. (2011). Bioengineering the infarcted heart by applying bio-inspired materials. *J Cardiovasc Transl Res* 4:559–574.
26. Leeper NJ, AL Hunter and JP Cooke. (2010). Stem cell therapy for vascular regeneration: adult, embryonic, and induced pluripotent stem cells. *Circulation* 122:517–526.

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