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Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair

David Zisa[#], Arsalan Shabbir[#], Gen Suzuki, and Techung Lee^{*}

Department of Biochemistry and Center for Research in Cardiovascular Medicine, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, USA

Abstract

We recently demonstrated a novel effective therapeutic regimen for treating hamster heart failure based on injection of bone marrow mesenchymal stem cells (MSCs) or MSC-conditioned medium into the skeletal muscle. The work highlights an important cardiac repair mechanism mediated by the myriad of trophic factors derived from the injected MSCs and local musculature that can be explored for non-invasive stem cell therapy. While this therapeutic regimen provides the ultimate proof that MSC-based cardiac repair is mediated by the trophic actions independent of MSC differentiation or stemness, the trophic factors responsible for cardiac regeneration after MSC therapy remain largely undefined. Toward this aim, we took advantage of the finding that human and porcine MSCs exhibit species-related differences in expression of trophic factors. We demonstrate that human MSCs when compared to porcine MSCs express and secrete 5-fold less vascular endothelial growth factor (VEGF) in conditioned medium (40 ± 5 and 225 ± 17 pg/ml VEGF, respectively). This deficit in VEGF output was associated with compromised cardiac therapeutic efficacy of human MSC-conditioned medium. Overexpression of VEGF in human MSCs however completely restored the therapeutic potency of the conditioned medium. This finding indicates VEGF as a key therapeutic trophic factor in MSC-mediated myocardial regeneration, and demonstrates the feasibility of human MSC therapy using trophic factor-based cell-free strategies, which can eliminate the concern of potential stem cell transformation.

Keywords

VEGF; mesenchymal stem cell; heart failure; cell therapy

INTRODUCTION

Clinical trials of mesenchymal stem cell (MSC) therapies are currently being conducted for treating a broad spectrum of diseases [1]. Remarkably, although the multi-lineage differentiation potential (stemness) of MSCs was originally thought to mediate their therapeutic attributes, it has now become clear that the secretion of multiple growth factors and cytokines

*Correspondence: Techung Lee, Department of Biochemistry, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, USA, Tel/ Fax: (716) 829-3106, chunglee@buffalo.edu.

[#]The two authors contributed equally to the manuscript.

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(trophic action) by MSCs is primarily responsible for many of the observed therapeutic benefits [2;3]. Consistent with this notion, studies have attributed the cardiac repair function of MSCs to their trophic actions independent of their differentiation potentials [4;5;6]. We have in particular demonstrated that while MSCs injected into the hind limb muscle are trapped in the local musculature with no detectable migration to the heart, the administered MSCs exert a prominent cardiac repair function in a hamster heart failure model, and this therapeutic effect can be reproduced with a cell-free strategy based on intramuscular injections of MSC-conditioned medium [7]. These studies together illustrate that the trophic mediators secreted by MSCs improve cardiac function by a combination of multiple mechanisms such as attenuating tissue injury, inhibiting fibrotic remodeling, promoting angiogenesis, mobilizing host tissue stem cells, and reducing inflammation, although the bio-active factors remain largely undefined.

While clinical trials of MSCs have demonstrated no significant adverse side-effects even with multiple cell administrations [1;8], long-term safety of MSC therapy remains to be evaluated. Notably, MSC transformation has been reported in prolonged ex vivo expansion [9;10;11], and over-expression of telomerase or inactivation of the Wnt pathway has been shown to provoke tumor formation from MSCs [12;13]. MSC abnormalities have also been detected in patients with multiple myeloma [14;15], suggesting that MSCs may be sensitive to precancerous or cancerous state of the host. Since MSC-derived trophic factors appear to underlie the cardiovascular therapeutic effects, they may be explored for development of trophic factor-based cell-free therapeutic strategies, thus eliminating the safety concern associated with cell transformation. Toward this end, we have begun to characterize the trophic actions of human and porcine bone marrow MSCs in relation to their therapeutic efficacy [7; 16;17]. We report here that cardiac repair mediated by the trophic actions of MSCs can be critically influenced by the ability of MSCs to express and secrete adequate levels of vascular endothelial growth factor (VEGF). This finding bears significance to development of MSC-based therapeutic regimens for cardiac regeneration.

METHODS AND MATERIALS

Animals

Bio-F1B (normal) and Bio-TO2 (cardiomyopathic) male hamsters were obtained from Bio Breeders (Watertown, MA). All procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

Echocardiography

Echocardiographic measurements were performed in a blind-folded fashion, and were described in our recent work [16;18]. Cardiac function was assessed by left ventricular ejection fraction (LVEF).

MSC culture

Fresh human bone marrows were purchased from Lonza Walkersville (Gaithersburg, MD). Human MSCs (hMSCs) were prepared and expanded as described for porcine MSC (pMSCs) [16;17;19]. hMSCs and pMSCs were culture-expanded in MesenPro/RS medium (Invitrogen) and DMEM/F12 medium containing 10% fetal bovine serum (FBS), respectively. Both media were supplemented with 2 mM glutamine, 50 µg/ml gentamycin, and 0.125 µg/ml Fungizone. Only MSC cultures exhibiting robust growth capacity (typically less than 5 passages) were used for the study. To generate MSC-conditioned medium, MSCs were grown on fibronectin-coated dishes to ~90% confluency. Cells were then washed thoroughly with Hank's balanced salt solution (HBSS), and maintained in serum- and phenol red-free Minimal Essential Medium

(MEM) for 24 hours. The conditioned medium was harvested, and filtered prior to use. The protocol for viral infection of hMSCs using Ad-VEGF165 was described previously [17].

Intramuscular injections of MSCs and MSC-conditioned medium

MSCs (2 – 4 million cells per animal) were resuspended in 1 ml HBSS, and injected in equally divided doses into the left and right hamstrings of 4-month old TO2 cardiomyopathic hamsters as described [16]. For saline or medium injection, each hamstring was injected with 0.5 ml of saline or conditioned medium two to three times a week for four consecutive weeks as described [16]. Echocardiography was performed 1 month after the initiation of the therapeutic injections.

Real time qRT-PCR

RNA isolation and qRT-PCR protocols were as described [17]. β 2-microglobulin (B2M) was used as the reference gene for calculations. Primer sequences are listed in Table 1.

ELISA Analysis

VEGF concentrations of MSC-conditioned medium were measured with a VEGF ELISA kit from R&D Systems (#DY293B DuoSet) following the manufacturer's protocol.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Comparisons between groups were based on Anova analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Preclinical and clinical studies of myocardial stem cell therapy often use invasive cell delivery approaches such as intramyocardial injection or intracoronary infusion, which can introduce harmful scar tissue, arrhythmia, calcification, or microinfarction in the heart [20;21;22;23]. Aiming at developing a safer cell delivery regimen and taking advantage of the trophic actions of MSCs, we recently demonstrated a prominent cardiac repair mechanism achieved by injections of pMSCs or pMSC-conditioned medium into the hind limb muscle of TO2 cardiomyopathic hamsters [7;18]. Intramuscularly injected pMSCs and pMSC-conditioned medium each significantly improved ventricular function one month post therapy. The functional improvement is associated with mobilization of bone marrow progenitor cells, increased myocardial capillary density, renewed cardiomyocyte populations, attenuated apoptosis, and decreased fibrosis [7].

We were particularly encouraged by the finding that intramuscular injections of pMSC-conditioned medium could achieve significant cardiac repair as this strategy could be further optimized for a trophic factor-based cell-free therapeutic regimen, eliminating the concern of potential MSC transformation after cell administration [10;11;12;13]. Since we noted species-related differences in the growth and trophic actions of MSCs during our characterizations of hMSCs and pMSCs [16], we first examined whether hMSCs delivered to the hind limb muscle of TO2 cardiomyopathic hamsters could also repair the failing hamster heart as demonstrated with pMSCs [7]. Figure 1A shows that hMSCs and pMSCs both significantly increased LVEF by ~30% four weeks after the cell therapy. We then compared the therapeutic potency of hMSC- and pMSC-conditioned medium (designated as hMSC CM and pMSC CM, respectively). Notably, although the injected hMSCs were therapeutically effective, hMSC CM failed to improve ventricular function of the failing hamster heart (Fig. 1B), suggesting that the hMSC-conditioned medium might not contain an adequate level of therapeutic trophic factors.

To further understand the difference in the trophic actions of hMSCs and pMSCs, we used qRT-PCR to quantify expression of growth factor and cytokine genes. Figure 2 shows that

among the seven genes analyzed pMSCs significantly expressed higher levels of insulin-like growth factor 1 (IGF1), monocyte chemotactic protein 1 (MCP1), and vascular endothelial growth factor-A (VEGF) than hMSCs. Since VEGF has been used in clinical trials for heart disease [24;25], the gene was singled out for further analysis. We further performed VEGF ELISA to validate the qRT-PCR data. Figure 3 shows that pMSC CM contained ~5 fold higher VEGF concentration than hMSC CM (225 ± 17 vs. 40 ± 5 pg/ml; $P < 0.001$), which is in agreement with the qRT-PCR data presented in Figure 2.

To determine whether the decreased VEGF output might be associated with the observed therapeutic deficit of hMSC CM, we overexpressed VEGF in hMSCs using a recombinant adenoviral vector as described previously [17;26], and prepared conditioned medium (designated as VEGF hMSC CM) from the VEGF-expressing hMSCs. Figure 3 reveals that infection of hMSCs by the VEGF adenoviral vector caused a pronounced increase in VEGF concentration of the conditioned medium ($10,118 \pm 210$ pg/ml). Injections of VEGF hMSC CM were subsequently found to completely restore the cardiac therapeutic potency of the hMSC-conditioned medium (Fig. 1B). Thus, although hMSCs exhibit a lower VEGF output compared to pMSCs, this deficit can be readily remedied by VEGF overexpression. These findings thus define VEGF as a key therapeutic trophic factor in MSC-mediated cardiac repair.

DISCUSSION

Despite recent recognition that the trophic actions of MSCs underlie their therapeutic effects in various preclinical and clinical studies [2;3;4], the trophic factors responsible for tissue functional improvement after MSC therapy remain largely undefined. While our study explored the feasibility of using a cell-free therapeutic strategy for cardiac repair based on MSC-conditioned medium, which can eliminate the concern of potential MSC transformation [9;11;12;13], it further demonstrated VEGF as a key therapeutic trophic factor in MSC-mediated cardiac repair. This demonstration is consistent with a recent report showing that MSC-derived VEGF plays a critical role in attenuating acute kidney injury [27].

Development of trophic factor-based cell-free therapeutic regimens for tissue repair can be facilitated through analysis of the trophic actions of MSCs as demonstrated here. Using a cocktail of well-defined trophic factors for this purpose is not only pharmaceutically sound but also strategically advantageous since the trophic factor cocktail can be precisely modified to treat different degenerative conditions. Our comparative study of pMSC CM, hMSC CM, and VEGF hMSC CM suggests that a cocktail with a VEGF dosage greater than ~200 pg/ml is sufficient to elicit significant cardiac repair for the failing hamster heart in conjunction with other yet undefined MSC trophic factors. This VEGF dosage amounts to ~2 ng VEGF/kg body weight per intramuscular injection, which is three orders of magnitude lower than the typical VEGF dosages used in single growth factor therapeutic trials (>10 μ g/kg body weight per injection) [28]. Interestingly, we have found that VEGF when administered alone (in the absence of MSC trophic factors) could also rescue the failing hamster heart at higher dosages (0.1–1 μ g/kg body weight per intramuscular injection) [29]. Despite repeated intramuscular injections, side effects such as tissue edema, angioma formation, and inflammation, which have been associated with VEGF administration [30;31], were not observed in our study. A prominent feature of the intramuscular VEGF or MSC delivery approach is that the local muscular bed produces additional beneficial soluble mediators in response to trophic factor signaling [7;29]. Activation of the host muscle trophic factor network thus further amplifies the trophic actions of MSCs, culminating in an effective cardiac repair mechanism.

MSCs are thought to have a perivascular origin, and can be derived from multiple human organs including bone marrow, skeletal muscle, placenta, pancreas, and adipose tissue [32]. It is presently unknown whether the therapeutic potency of MSCs may be affected by the tissue

source. In transitioning toward the clinical use of hMSCs, additional variables such as differences in donor gender, age, and life style can potentially complicate the logistic aspect of MSC therapy. For instance, limited information indicates that female stem cells may possess a more pronounced regenerative potential than male stem cells [33], which is in line with the finding that female patients typically exhibit certain cardioprotective phenomenon from acute myocardial infarction and better outcome after the incidence compared to male patients [34]. Although these gender influences are thought to be mediated through differential sex hormone receptor signaling, a recent study shows that female rodent MSCs produce a higher level of VEGF than male rodent MSCs in response to hypoxia [35]. Our demonstration here that VEGF hMSC CM, but not hMSC CM, improved cardiac function further highlights the critical role of VEGF in mediating the therapeutic function of MSCs. We suggest that the cardiac therapeutic potency of a given donor MSC may correlate with the cell's ability to express and secrete an adequate level of VEGF, which can be readily assessed by qRT-PCR and ELISA as shown here.

The cardiovascular effects of VEGF appear to stem from its multiple biological attributes. VEGF is well known for its ability to mobilize bone marrow progenitor cells, and these recruited progenitor cells can participate in myogenesis and angiogenesis [36]. In addition to being an angiogenic growth factor, VEGF also possesses a nonangiogenic role important for the cardiovascular system [37]. VEGF can promote differentiation of stem cells into cardiomyocytes and endothelial cells [38;39], and boost the function of MSCs and endothelial progenitor cells [40;41]. We have demonstrated that MSCs engineered to overexpress VEGF exhibit more pronounced trophic factor outputs [17]. These trophic factors acting in concert can mediate cardiac repair through their progenitor cell-mobilizing, angiogenic, cytoprotective, myogenic, anti-fibrotic, and anti-inflammatory properties [7;16]. Interestingly, an MSC-derived anti-inflammatory factor designated as TSG-6 has been found to mediate cardiac repair after myocardial infarction [6]. Given that tissue damage and degeneration is complex in nature, multiple MSC trophic factors acting in synergy are expected to be involved in tissue healing. Identification of these trophic factors can eventually lead to the development of cell-free trophic factor cocktails suitable for various clinical applications.

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Figure 1.

Left ventricular functional improvement mediated by human and porcine MSCs and MSC-conditioned medium. Echocardiography was performed in a blind-folded manner. Left ventricular ejection fraction (LVEF) was measured pre-injection, 2 weeks, and 4 weeks after initiation of injections. As reference, normal age-matched F1B hamsters maintain a stable LVEF of ~70%. Panel A: TO2 hamsters were injected with hMSCs and pMSCs. A saline injection control group was also included. Panel B: TO2 hamsters were injected with MSC-conditioned medium. Conditioned medium was also prepared from hMSCs overexpressing VEGF (designated as VEGF hMSC CM). n=6 per animal group. Anova analysis was performed for group comparisons. *P<0.005 vs. pre-injection; %P<0.05 vs. pre-injection; #P<0.005 vs. saline control.

Figure 2.

Expression profiles of hMSC and pMSC trophic factors analyzed by qRT-PCR. Primer sequences were listed in Table 1. Total RNA isolated from hMSCs and pMSCs was analyzed by qRT-PCR. Threshold cycles were normalized based on the reference gene β 2-microglobulin (B2M). n=3. *P<0.05 between hMSCs and pMSCs.

Figure 3.

Measurement of VEGF concentrations by ELISA. Conditioned medium was prepared from hMSCs, pMSCs, and hMSCs overexpressing VEGF. The medium was assayed by a VEGF ELISA kit. VEGF concentrations were expressed as pg/ml shown above each bar. n=3.

*P<0.001 compared to hMSC CM.

Table 1

Gene	Forward Primer	Reverse Primer
Human B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
Human FGF2	ACCCGACGGCCGA	TCTTCTGCTTGAAGTTGTAGCTTGA
Human IGF1	CAGCAGTCTTCCAACCCAAT	ACAGCGCCAGGTAGAAGAGA
Human IL6	CGGGAACGAAAGAGAAGCTCTA	GGCGCTTGTGGAGAAGGAG
Human LIF	ACCAGATCAGGAGCCAACTG	GCCACATAGCTTGTCCAGGT
Human MCP1	CATTGTGGCCAAGGAGATCTG	CTTCGGAGTTTGGGTTTGCTT
Human TGFb	CGAGAAGCGGTACCTGAAC	TGAGGTATCGCCAGGAATTGT
Human VEGF-A	GCACCCATGGCAGAAGG	CTCGATTGGATGGCAGTAGCT
Gene	Forward Primer	Reverse Primer
Porcine B2M	AAACGGAAAGCCAAATTACC	ATCCACAGCGTTAGGAGTGA
Porcine FGF2	GGAGTGTGTGCAAACCGTTA	TCGTTTCAGTGCCACATACC
Porcine IGF1	TGCTCTCCTTCACCAGCTCT	GCCTCCTCAGATCACAGCTC
Porcine IL6	TTCACCTCTCCGGACAAAAC	TCTGCCAGTACCTCCTTGCT
Porcine LIF	GTCACCCATGTCACAGCAAC	CCCCTGGGCTGTGTAGTAGA
Porcine MCP1	CACCAGCAGCAAGTGTCTTA	TCCAGGTGGCTTATGGAGTC
Porcine TGFb	AAGCGGCAACCAATCTATG	CACGTGCTGCTCCACTTTTA
Porcine VEGF-A	CTACCTCCACCATGCCAAGT	ACACAGGACGGCTTGAAGAT