Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment.

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Abstract

The aim of this study was to determine whether intravenously-administered multipotent stromal cells from human bone marrow (hMSCs) can improve cardiac function after myocardial infarction (MI) without long-term engraftment and therefore whether transitory paracrine effects or secreted factors are responsible for the benefit conferred. hMSCs were injected systemically into immunodeficient mice with acute MI. Cardiac function and fibrosis after MI in the hMSC-treated group was significantly improved compared with that in controls. However, despite the cardiac improvement, there was no evident hMSC engraftment in the heart 3 weeks after MI. Microarray assays and ELISAs demonstrated that multiple protective factors were expressed and secreted from the hMSCs in culture. Factors secreted by hMSCs prevented cell death of cultured cardiomyocytes and endothelial cells under conditions that mimicked tissue ischemia. The favorable effects of hMSCs appear to reflect the impact of secreted factors rather than engraftment, differentiation, or cell fusion.

Keywords

multipotent stromal cells; myocardial infarction; cellular engraftment; secreted factors; cytoprotection

Cell-based therapy is a promising approach for treatment of myocardial infarction (MI) [1]. Bone marrow-derived cells have been the focus in several experimental animal studies and clinical trials [1], [2] and [3].

Bone marrow contains adult stem/progenitor cells for non-hematopoietic tissues that are isolated primarily by their adherence to tissue culture plastic. The cells have been variously isolated...
referred to as fibroblastoid colony forming units, mesenchymal stem cells and, more recently as multipotent stromal cells or MSCs [4]. MSCs can be isolated easily by bone marrow aspiration, expand extensively in culture, and differentiate into multiple cell phenotypes \textit{ex vivo} and \textit{in vivo}. Although the cardiomyogenic potential of hematopoietic stem cells has been questioned [5] and [6], MSCs can express phenotypic characteristics of cardiac myocytes [7]. Thus, the ability of engrafted MSCs to differentiate into cardiac lineage cells has been explored [3], [8], [9], [10] and [11]. However, it is not clear whether long-term engraftment and differentiation of MSCs in the heart are necessary for improvement of cardiac function or alternatively whether the benefits can be conferred by transitory paracrine effects or secreted factors. Accordingly, we performed the present study to determine whether intravenously-administered hMSCs engraft in the immunodeficient mouse heart after MI and whether they improve cardiac function consequently or independently of persistent engraftment. Immunodeficient (NOD/SCID/beta2-microglobulin$^{-/-}$) mice were used because they have reduced innate immunity, lack natural killer cell activity, and are suitable for xenotransplantation of human cells [12].

The therapeutic potential of secreted factors from adult stem/progenitor cells has been recognized [13], [14], [15], [16] and [17]. Accordingly, we evaluated the gene expression profiles for proteins implicated as potential antiapoptotic, angiogenic/arteriogenic and matrix-mediating factors in cultured hMSCs. We compared the gene expression profiles of cultured hMSCs with those of freshly isolated CD133-positive bone marrow stem cells currently being evaluated as a candidate cell population for treatment MI in patients [18]. To clarify the implications of the array data, we ran ELISAs for several protective secreted factors on serum-free conditioned medium (CdM) from the hMSC donor that was used to treat the mice with MI. CdM from the hMSCs rescued both murine cardiomyocytes (HL-1 cells) and human umbilical vein endothelial cells (HUVECs) from cell death during hypoxia exposure.

Materials and methods

Preparation of cells

Human MSCs were obtained from the Tulane Center for the Preparation and Distribution of Adult Stem Cells (http://www.som.tulane.edu/gene_therapy/distribute.shtml). Passage 1 hMSCs were transduced with lentivirus containing humanized Renilla green fluorescence protein (hrGFP) under control of the CAG promoter (lentiviral vector modified from the Trono Lab, Switzerland).

Induction of MI in mice, transplantation of hMSC, and echocardiography

NOD/SCID/beta2-microglobulin(null) mice were ventilated mechanically under anesthesia, the chest was opened, and the left anterior descending coronary artery was ligated. Randomly selected mice underwent 3 systemic administrations of hMSC or vehicle weekly at 1, 8 and 15 days after MI. In the hMSC-treated group, 2 to 5 x 10$^6$ cells in 0.1 ml of Hank’s Balanced Salt Solution (HBSS) was injected into mice via the tail vein. Controls were given the same volume of HBSS. Mice that survived three weeks after MI (MSC, n=5; control, n=4) were studied.

Echocardiography was performed 1, 2 and 3 weeks after MI by an investigator who was blinded to the experimental protocols. The following variables were assessed: left ventricular diameter in end-diastole (LVDd), LV diameter in end-systole (LVDs), LV anterior wall thickness in end-diastole (LVAWth), and LV posterior wall thickness in end-diastole (LVPWth). The percentage of fractional shortening (%FS) was calculated automatically.
Detection of hMSCs engraftment

To detect hMSCs engraftment, immunohistochemistry for GFP, conventional PCR for human-specific mitochondrial cytochrome oxidase I (HmitoCOX-1) and real-time PCR for human Alu sequences in mouse heart were performed.

Conditioned medium protection experiments in hypoxia

For protection experiments, both HL-1 cardiomyocytes and HUVECs were plated separately at 500 cells/cm$^2$ in their appropriate growth media. Three days after plating, the medium was replaced with either CdM (10x) or fresh serum-free medium (SFM) and the cells were rendered hypoxic (1% oxygen) in a specialized incubator for 18 hrs. Cells cultured under normoxia for 18 hrs in the growth medium were used as additional controls. After 18 hrs, the cells were lifted by trypsin/EDTA and were quantified by fluorescent labeling of nucleic acids (CyQuant; Molecular Probes).

Statistical analysis

Values were expressed as means ± SEM unless indicated otherwise. Comparisons among the three groups were made with the use of one-way analysis of variance (ANOVA) followed by Scheffé’s multiple comparison test. Comparisons between two groups were made with the use of unpaired Student’s $t$-tests. $P < 0.05$ was considered significant.

An expanded Materials and Methods section can be found in an online data supplement.

Results

Effects of transplantation of hMSC on LV structure and function

MIs were induced in the mice, and hMSCs were infused intravenously after 1, 8 and 15 days. M-mode echocardiographic images 3 weeks after MI showed that both the control and hMSC-treated mice compared with sham operated animals exhibited impaired contractility and LV dilation. However, both were less prominent in the group of animals that had been given hMSCs (Fig. 1A). Three weeks after MI the %FS in the hMSC-treated group was significantly higher than in the control group (p<0.05, Fig. 1B). Administration of hMSCs reduced the progression of LV cavity dilation and wall thinning measured in end-diastole compared with values in controls (Fig. 1B).

To examine the time course of effects after treatment with hMSCs, mice were assessed at 1, 2 and 3 weeks after MI. Significant differences in %FS were maintained between control and hMSC-treated animals throughout the experimental period and were persistent 3 weeks after MI (Supplementary Fig. S1A). LVDd was significantly greater in control compared with hMSC-treated animals 1 week after MI but not 2 or 3 weeks after MI (Supplementary Fig. S1B). Thus, the echocardiographic studies showed that the systemic administration of hMSCs via intravenous injection significantly improved cardiac function after MI.

Reduction of cardiac fibrosis in mice given hMSCs intravenously

Trichrome-stained sections of hearts from mice given hMSCs showed a less enlarged LV cavity and less fibrosis than those from controls at 3 weeks after MI (Fig. 2A, upper panels). In H&E-stained sections greater wall thickness and cellular density were observed in the zone of infarction in the hMSC-treated mice (Fig. 2A, lower panels). Quantitative analysis demonstrated that the percentage of fibrotic area was significantly less in the hMSC-treated mice compared with controls (p<0.05, Fig. 2B).
Lack of long-term engraftment of hMSCs in hearts subjected to infarction

The extent of engraftment of hMSCs was assessed by immunohistochemistry to detect GFP, real-time PCR to detect human Alu sequences, and conventional PCR to detect HmitoCOX-1. As judged by immunohistochemical detection of GFP, there were no human cells in the hearts of any of the hMSC-treated mice at 3 weeks after MI (n=5; see representative images, Fig. 3A).

Conventional PCR to detect the HmitoCOX-1 gene is a sensitive assay for detection of engraftment because the primer sequences that we used are specific for human mtDNA and do not amplify mtDNA from mouse. Furthermore, the copy numbers of mtDNA in human cells frequently range in the thousands [19]. However, we could not detect any amplification of the HmitoCOX-1 gene from DNA from the hearts of mice treated with hMSCs (Fig. 3B).

Real-time PCR for detection of human Alu sequences is very sensitive [17]. It permits recognition of engraftment of human cells; this assay can detect 0.5 pg of human DNA in 300,000 pg of mouse DNA. Thus, the assay can theoretically detect the presence of 1 human cell among 600,000 cells in the mouse heart. However, with the use of this sensitive assay, no human DNA was detectable in the hearts of any of the mice subjected to infarction (data not shown). A previous study reported an estimate of the number of cardiomyocytes in an adult murine heart as \( \sim 3 \times 10^6 \) [20]. As judged from this estimate, less than 5 hMSCs engrafted and survived long term in the mouse hearts subjected to infarction in our study. Accordingly, with the use of 3 different assays for detection of hMSC engraftment, no human cells were detectable in the mouse hearts 3 weeks after MI in the present study. Even if a few hMSCs did engraft and survive in the hearts with infarction, the numbers would be far too low to account for the improved cardiac function that we observed.

Expression of genes encoding secreted factors in hMSCs compared with freshly isolated CD133(+) bone marrow-derived stem cells

Because of the lack of persistent engraftment of hMSCs administered intravenously in mouse hearts subjected to infarction, we hypothesized that their favorable impact on cardiac function reflected the impact of transitory paracrine effect or those of factors secreted in vivo by the cells. Accordingly, we assayed the gene expression profiles of cultured hMSCs using microarrays. As a reference, we compared the data with microarray assays of freshly isolated CD133(+) bone marrow cells known to contain hematopoietic and endothelial stem/progenitor cells (Supplementary table 1). The cultured hMSCs expressed mRNAs for anti-apoptotic, angiogenic/arteriogenic, and matrix-mediating factors. Many of the mRNAs for secreted proteins were expressed to a greater extent in the hMSCs than in the freshly isolated CD133(+) cells. In particular, expression of IL-6, leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) family members in the hMSCs was 40 to 200 fold higher than in the CD133(+) cells (Supplementary table 1). Several mRNAs for matrix-mediating factors such as matrix metalloproteinase (MMP)-2, inhibitors such as (TIMP)-1,2, and matricellular proteins (Thrombospondin-1 and Tenascin C) were also highly expressed in hMSCs as well (Supplementary table 1). The results of the microarray analysis demonstrated that cultured hMSCs expressed mRNAs for a variety of secreted factors that may be cardioprotective and reparative.

Protective factors secreted by hMSCs

In confirmation of several transcripts by the microarray assays, ELISAs of serum-free medium conditioned by 90% confluent hMSCs for 48 hrs contained secreted VEGF, hepatocyte growth factor (HGF), adrenomedullin, placental growth factor (PLGF) and IL-6. Among these factors, adrenomedullin was secreted at the highest levels per total protein in the medium (Fig. 4A).
Protection of cardiomyocytes and endothelial cells by CdM from hMSCs

To elucidate the therapeutic potential of factors secreted by the hMSCs used in our in vivo study, we employed an ex vivo model mimicking ischemia (nutrient deprivation and hypoxia). Concentrated CdM (10 x) from hMSCs significantly prevented cell death for both HL-1 cardiomyocytes and HUVECs under hypoxia (HL-1, p<0.0001; HUVEC, p<0.0001; Fig 4B). Thus, these data suggest that factors secreted by hMSCs can protect both cardiomyocytes and endothelial cells from cell death caused by ischemia.

Discussion

MSCs possess properties that may ultimately allow their use in safe, convenient and effective cell therapy for MI. Our echocardiographic results demonstrated that treatment with systemically-administered hMSCs can ameliorate cardiac dysfunction after MI in immunodeficient mice. Cell administration also reduced the extent of fibrosis 3 weeks after MI. Because the mice that we used were immunodeficient, it is not likely that immunosuppressive effects of systemically-administered hMSCs would account for their favorable impact on cardioprotection or repair.

The favorable effects of MSCs on jeopardized myocardium are consistent with previous observations [3], [8], [9], [10], [11], [13] and [14]. However, the mechanisms by which systemic administration of the cells exerts such effects have not previously been elucidated. Previous studies have focused on the capacity of engrafted MSCs to differentiate into cardiac lineage cells [3], [8], [9], [10] and [11]. However, despite the use of three different and highly sensitive assays for human cells, we found that hMSCs administered intravenously once a week for 3 weeks did not stably engraft into hearts subjected to infarction. Although we cannot rule out short term, transient hMSC engraftment or transfer of mitochondria to injured cardiac myocytes [21], the injected hMSCs in our study were most likely to have been trapped in the lung vascular tree or elsewhere. This possibility is supported by results in previous studies that have tracked the distribution of MSCs after intravenous infusion [22] and [23]. Thus, others have observed with uninjured immunodeficient mice that hMSCs administered by intravenous injection are trapped in the lung and are undetectable in the heart 1 hour or 4 weeks after injection [22]. Similar results have been obtained in rats subjected to MI [23].

Our results with microarrays indicate that hMSCs are a robust source of secreted factors compared with freshly isolated CD133(+) bone marrow cells. In patients, intracoronary injections of freshly isolated CD133(+) cells [18] or endothelial progenitors [24] after acute MI have resulted in improved LV function. Such effects may be attributed to secreted factors [5] and [16]. In our study, hMSCs compared with CD133(+) cells were found to express higher mRNA levels for IL-6, LIF and VEGF family members. Several of these factors can prevent cell death of cardiomyocytes and endothelial cells [25] and [26]. Adrenomedullin mRNA was highly expressed by hMSCs. This peptide appears to be a powerful angiogenic and cardioprotective factor [27]. Consistent with our microarray results, CdM generated from the hMSCs of the donor whose cells improved cardiac function in mice with MI contained secreted adrenomedullin, VEGF, HGF, PLGF, and IL-6. In support of the hypothesis that factors secreted by the cells in vivo were likely responsible for improvements in cardiac function in the absence of hMSC engraftment, concentrated CdM from the same hMSC donor rescued significant numbers of both murine cardiomyocytes and human endothelial cells from cell death during 18 hrs of hypoxia exposure ex vivo. Thus, it appears that the beneficial effects of the hMSC administration come from cardioprotection by transitory paracrine effects or effects of secreted factors in the acute phase after MI.

Gnecchi et al. [13] and [14] reported that conditioned medium from genetically-modified rat MSCs overexpressing Akt prevented cardiomyocyte death. The present results corroborate and
extend the findings from those reports to human MSCs. In summary, the present results demonstrate that hMSCs can exert favorable cardiac effects after MI without persistent engraftment, differentiation, or cell fusion. Thus, the mechanisms responsible for the benefits appear to be attributable to transitory paracrine effects or secreted factors following systemic administration of the cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.
Echocardiographic data obtained 3 weeks after myocardial infarction (MI). (A) Representative M-mode echocardiograms obtained from Sham (left), Control group (center), and hMSC-treated group (right). Arrows show left ventricular (LV) chamber. (B) Individual variables in M-mode. %fractional shortening (%FS), LV diameter in end-diastole (LVDd), LV diameter in end-systole (LVSd), LV anterior wall thickness in end-diastole (LVAWth), LV posterior wall thickness in end-diastole (LVPWth). Data are means ± SEs, n=4 in control group, n=5 in hMSC-treated group, n=3 in sham group, † p < 0.05 compared with sham, * p < 0.05 compared with controls.
Figure 2.
Results of histological assessments at 3 weeks after MI. (A, upper panels) Trichrome-stained sections (magnification x20). (A, lower panels) H&E-stained sections (magnification x100). (B) Comparison of % fibrotic area in heart with infarction in control and hMSC-treated mice. Data are means ± SEs. n=4 in control group, n=5 in hMSC-treated group, * p< 0.05 compared with controls.
Figure 3.
Lack of engraftment of hMSCs at 3 weeks after MI. (A) Representative sections showing immunofluorescence for α-sarcomeric actin (green) and GFP (red). No GFP-positive cells are engrafted into myocardium or blood vessels in the border zone of the infarction. I, infarct area; M, myocardium; CA, coronary artery. (B) PCR for human-specific mitochondrial COX-I gene. The PCR product could not be amplified from DNA samples that were isolated from the hearts of 5 mice treated with hMSCs given intravenously after acute MI. N, water; mHeart, mouse heart DNA.
Figure 4.
Characterization and effects of factors secreted by hMSCs. (A) Factors secreted into serum-free medium by hMSCs in culture. ELISAs were performed in triplicate on medium that was concentrated 10 fold by diafiltration. Data are expressed as ng of protein per total mg protein as determined by protein assay (Biorad DC protein assay). VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; PLGF, placental growth factor; AdM, adrenomedullin. (B) Serum-free conditioned medium (CdM) protects against hypoxia exposure. Rescue of HL-1 murine cardiomyocytes (left) and HUVECs (right) by CdM (10 x). Both cell types were exposed to hypoxia (1% O₂) and in CdM or SFM for 18 hrs. GM= growth medium. SFM= fresh serum-free medium. Data are means ± SE, n=3. ** p< 0.0001 compared with SFM.