Mobilization and homing of bone marrow stromal cells in myocardial infarction

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Abstract

Objective: Marrow stromal cells (MSCs) contain multipotent cells, which may participate in the repair of damaged organs. We tested the hypothesis that MSCs are recruited to the heart upon myocardial infarction (MI), and play pathophysiological roles in the healing and adaptation process.

Methods: Donor MSCs from isogenic Lewis rats were harvested, multiplied and labeled with Lac Z reporter gene. Ten million labeled cells were injected intravenously into the recipient rats (n=30). One week later, 10 rats were killed to examine the distribution of the labeled MSCs. Other rats underwent either coronary artery ligations (n=14) or sham operations (n=6). The hearts were removed at various time points (1–8 weeks) and stained for β-galactosidase activity. Phenotypes of labeled cells were identified with immunohistochemical stains.

Results: In rats killed at 1 week, labeled cells had homed into the bone marrow of the recipients, and none found in their hearts. In the coronary ligated hearts, labeled cells were seen in and near the infarct at all time points studied (14/14), but none in the sham operated hearts (6/6). There was evidence for myogenic differentiation. Some of these labeled cells showed positive staining for cardiomyocyte specific troponin I-c at 4 weeks, while others appeared in the vascular walls expressing smooth muscle alpha-actin.

Conclusions: Following myocardial infarction, MSC’s are signaled and recruited to the injured heart, where they undergo differentiation, and may participate in the pathophysiology of post-infarct remodeling, angiogenesis, and maturation of the scar. Therapeutic implantation of MSCs thus may further enhance such effects.

Keywords: Myocardial infarction; Adult stem cells; Physiology; Myocytes; Angiogenesis

1. Introduction

There is increasing evidence that bone marrow stroma contains pluripotent stem cells and progenitor cells, which can differentiate into cells of various phenotypes in the myocardium, and participate in myogenesis and angiogenesis [1,2]. Direct implantation of marrow stromal cells (MSC) into myocardium with infarction [3], or cytokine induced recruitment of MSCs into circulation prior to myocardial infarction [4], had been reported to improve cardiac functions. In this study, we tested the hypothesis that recruitment of MSCs from bone marrow, which traffic to the myocardial infarct site to differentiate and participate in the post-infarct repair, is a part of usual pathophysiology in myocardial infarction. Thus we posit that inflammatory response seen after myocardial infarction involves not only scavenger cells, which remove tissue debris, but also marrow-derived adult stem cells and progenitor cells, which can participate in the post-infarct repair process, such as angiogenesis [5], scar maturation [6], myogenesis and remodeling.

2. Methods

2.1. Animals

Male isogenic Lewis rats were obtained from Charles River laboratories. All animals received humane care in compliance with the ‘Guide to the Care and Use of Experimental Animals’ of the Canadian Council on Animal Care.
2.2. Isolation and culture of marrow-derived stromal cells

Isolation and primary culture of MSC’s from the femoral and tibial bones of donor rats were performed according to Caplan’s method [7]. The femoral and tibial bones were collected and both ends of the long bones were cut away from the diaphyses. The bone marrow plugs were hydrostatically expelled from the bones with complete medium, consisting of Dulbecco’s Modified Eagle’s Medium (DMEM) containing selected lots of 10% calf serum and antibiotics (100 U/ml penicillin G, 100 μg/mg streptocycin, 0.25 μg amphotericin B, all obtained from Gibco laboratories) in a humidified atmosphere of 5% CO₂. The marrow plugs were disaggregated and the dispersed cells were centrifuged and resuspended twice in complete medium. These cells in 10 ml of complete medium were then introduced into tissue culture dishes. Medium was replaced every 3 days and the non-adherent cells discarded. Each primary culture was twice divided into three new plates and cultured until the cell density of the colonies grew to approximately 90% confluence. They were then labeled as follows.

2.3. MSCs labeling with LacZ

LacZ-GP + AM12 amphotropic retrovirus producer cells were obtained from Dr Jacques Galipeau’s Laboratory (McGill University, Lady Davis Institute for Medical Research, Montreal, QC, Canada) [8]. These cells produce a replication defective retrovirus containing the LacZ gene that encodes for the bacterial β-galactosidase enzyme. These cells were cultured in DMEM with 10% FBS and antibiotics (50 U/ml Penicillin G and 50 μg/ml Streptomycin from Wisent Inc.). The cells were allowed to proliferate until at least a 70% confluence was achieved before using them for transduction in order to achieve a high retrovirus titer. Twenty-four hours before transduction, the marrow stromal cells were trypsinized with 0.05% Trypsin + 0.53 mM EDTA (Gibco Labs) and replated. The next day, these cells were transduced with LacZ retroviral particles twice per day for 3 consecutive days with lipofectamine 2 mg/ml solution for each 1 ml of virus medium. At each transduction, the MSC medium was replaced with the supernatant from the LacZ-GP + AM12 cells (after being filtered through a 0.45-μm filter). Seventy-two hours after the last transduction, the marrow stromal cells were trypsinized and part of the cells were plated in a 35-mm dish for histochemical staining for β-galactosidase activity in order to determine the percentage of cells expressing β-gal activity. The medium was aspirated from the plates and the cells rinsed with phosphate buffered saline (PBS). The cells were fixed at 4 °C in fix solution (2% formaldehde and 0.2% glutaraldehyde in PBS) for 15 min and re-rinsed with PBS. Staining for β-gal activity was performed with a solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), 2% dimethylsulfoxide, 20 mM K₃Fe(CN)$_₆$, 20 mM K₄Fe(CN)$_₆$·3H₂O and 2 mM magnesium chloride. The cells were then incubated at 37 °C, pH 7.8 and protected from light for 16 h. The presence of blue-labeled cells was then confirmed under phase microscopy.

2.4. Preparation of cells for injection

Cells isolated from the bone marrow were cultured in complete medium in tissue culture dishes. The medium was aspirated and the cells in each dish were washed with 6 ml of Hank’s Basic Salt Solution (HBSS). The HBSS was aspirated and 2 ml of trypsin-EDTA was added to detach the cells from the bottom of the dish. The detached cell suspension was then placed in a flask with 2 ml of complete medium and placed in a hemocytometer for counting; 5 × 10⁶ cells was then collected and centrifuged at 2500 rpm for 5 min, and the cell pellet resuspended in 0.5 cm³ of complete medium.

2.5. Intravenous injection for implantation of MSC’s

The recipient rats underwent intravenous injection of labeled isogenic MSC’s. The recipient Lewis rat was placed in a glass canister and 3% isoflurane at 1.5 l/min was introduced until anesthesia was achieved. The rat was then removed and placed in a restraining device and intubated with a 16-gauge intravenous catheter and ventilated at a tidal volume of 2.5 ml and a respiratory rate of 85 breaths/min; 5 × 10⁶ labeled cells suspended in 0.5 cm³ of DMEM were injected into the penile vein with a 28.5-gauge needle; 5 × 10⁶ labeled cells were re-injected in the same manner 24 h later.

2.6. Experimental groups

2.6.1. Marrow transplant series (n10)

The presence of labeled MSCs within the recipients’ bone marrow following the procedure described above was confirmed at various time points ranging from 2 h to 6 weeks later. The bone marrow was harvested as described earlier, plated and cultured in a similar fashion. When the cells were approximately 50–60% confluent, they were stained for β-gal activity as described above.

2.6.2. Coronary artery ligation versus sham operation series

One week after intravenous MSC injection, the rats underwent either coronary artery ligation (n = 14), or sham operation (n = 6), using the technique described below.

2.7. Operative procedures

The recipient rats were anesthetized with isoflurane (MTP Pharmaceuticals). They were intubated and ventilated at 85 breaths/min. The heart was exposed via a left
thoracotomy incision. For the coronary artery ligation group, the left coronary artery was ligated proximally using a 7-0 polypropylene suture. Sham operated rats were anesthetized and ventilated in a similar manner, and underwent a left thoracotomy without coronary artery ligation.

2.8. Histology and histochemical staining for β-galactosidase activity

The rats were killed at various time points: 1 week/three rats; 2 weeks/three rats; 4 weeks/six rats; 6 weeks/five rats; 8 weeks/three rats. The hearts were harvested and rinsed with PBS and perfusion fixed in 2% paraformaldehyde in PBS. The staining for β-galactosidase activity was performed as described above, but with the addition of 0.02% Nonidet P-40 and 0.01% deoxycholate to the staining solution. The gross heart specimens were stained for 6 h at 37°C and pH 7.8. After X-gal staining, they were embedded in paraffin and coronal sections 5 μm thick were mounted on a set of gelatin coated glass slides such that serial sections could be used for different stains. A series of sections from each heart specimen were stained with hematoxylin and eosin and another series stained with Picrosirius Red stain for connective fibrous tissue. Other serial sections from each heart were used for immuno-histochemical stainings, for sarcomeric myosin heavy chain molecules with MF20 (Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences); for connexin 43 (Zymed Laboratories Inc, San Francisco, CA); for troponin I-c (Santa-Cruz Biotechnology Inc); and for alpha smooth muscle actin (Sigma Laboratories). Briefly, after de-paraffinization, sections were placed in boiled citrate buffer (pH 6.0). After blocking in normal serum, sections were treated with the respective monoclonal antibodies overnight and with secondary antibody the following day. Diaminobenzidine (DAB), which produces brown color was then used as a chromogen for light microscopy. Counter-staining of sections by hematoxylin and eosin was also performed. Cells derived from labeled MSC’s were identified by their blue nuclei.

3. Results

The MSC’s in culture were observed under phase microscopy and assessed for proliferation and morphology at each culture medium change. The hematopoietic cells were not adherent and mostly removed after the second change of medium. After approximately three to four passages, the cells were expanded to over 25.0 million cells from the initial 250–500, which had adhered to the bottom of the culture dish. The transfection efficiency for cell labeling was nearly 100%, and labeled cells stained with X-gal solution in the culture dish had blue nuclei when viewed under phase microscopy (Fig. 1).

Intravenous implantation of cells on two consecutive days yielded no mortality and the rats tolerated the procedure well. The homing of labeled cells into the bone marrow of the recipient rats after implantation was confirmed in the ‘Marrow transplant series’ at various time points. In all rats, both in the sham operated and coronary artery ligated groups, the harvested bone marrow contained 1–2% labeled cells within each culture plate.

Fig. 1. Labeled MSC’s prior to implantation in culture dish. Note 100% transfection efficiency.

Fig. 2. Gross heart specimen after coronary ligation at 4 weeks stained with X-gal. Note blue color in scar distribution from MSC migration.
In the ‘Coronary artery ligation versus sham operation series’ of experiments, all rats survived the coronary artery ligation or sham operations until the time of killing. Gross examination of the coronary ligated hearts revealed a scar in the distribution of the left anterior descending coronary artery in all hearts. The hearts were stained with X-gal solution and gross examination revealed blue discoloration localized in the area of scarred myocardium in all coronary ligated hearts (Fig. 2). The adjacent normal myocardium remained unstained, indicating the presence of labeled MSC’s only in the territory of the infarcted myocardium (Fig. 3). The sham operated hearts also underwent X-gal staining, which revealed the absence of any blue color in the hearts.

When serial coronal cross-sections of the hearts were examined, labeled MSC’s could be identified in all 14 coronary ligated hearts in the region of the scar, and in none of the six sham operated hearts. Sections stained with cardiomyocyte specific troponin I-c antibody. Brown color in the cytoplasm indicates positive stain.

![Fig. 3. Oblique view of gross heart specimen in Fig. 2. Arrows denote border zone between scar and normal myocardium, showing absence of blue color in the non-infarcted posterior myocardium.](image)

![Fig. 4. Cross-section of Picrosirius Red stained heart in Fig. 2 through scar region. Arrows denote labeled MSC’s.](image)

![Fig. 5. Cross-section of myocardial scar 6 weeks after coronary ligation, stained with cardiomyocyte specific troponin I-c antibody. Brown color in the cytoplasm indicates positive stain.](image)

![Fig. 6. Cross-section of an arteriole in a region of myocardial scar 6 weeks after coronary ligation. A labeled MSC can be found in the wall of the arteriole, positive for immunohistochemical stain for alpha smooth muscle actin (brown). Counterstained with H and E.](image)
Picrosirius Red to visualize connective and fibrous tissue showed labeled MSC’s localized within the fibrous tissue regions, which stained red, and not in the adjacent normal myocardium, which stained yellow (Fig. 4). Labeled MSC’s were evident in infarcted myocardium at all time points studied. Up until 3 weeks, the MSC-derived cells showed an immature monocyte-like appearance with a large nucleus to cytoplasm ratio. Heart specimens obtained and studied at all earlier time points did not reveal clear evidence of differentiation. By 3 weeks after coronary artery ligations, immunohistochemical staining showed evidence for cardiac-myogenic differentiation in some labeled cells with the presence of the cardiomyocyte specific protein [9], troponin I-c. This staining for troponin I-c was more pronounced in the cytoplasm of these cells by 4–6 weeks, with further morphologic changes indicative of cardiomyogenic differentiation at 6 weeks (Fig. 5). These cells appeared more elongated in shape, and aligned with adjacent labeled cells. Connexin 43, the main constituent protein in the cardiac gap junctions [10] was evident as early as 4 weeks. Sections stained for alpha smooth muscle actin identified the differentiation of labeled MSC’s into smooth muscle phenotype, with some of them appeared to have been incorporated into the walls of arterioles (Fig. 6), suggesting a role in angiogenesis.

4. Discussion

Findings reported here indicate acute myocardial infarction can recruit MSCs from the bone marrow. Although the signaling mechanism is uncertain, cytokines from the infarct site may play a role, since earlier studies showed that exogenous cytokines such as GM-CSF can enhance the mobilization of pro-endothelial cells [11] and myogenic cells [4] from the bone marrow. On the other hand, mobilization and trafficking of marrow-derived cells to the heart had also been reported in adult dystrophic mdx mice [12], as well as in human heart transplant patients [13], and it is not clear whether cytokines play any signaling roles in such cases. Our study indicates that although these cells will differentiate and survive long term in the heart, the process of recruitment of MSCs to the heart occurs early, i.e. within days following myocardial infarction, and is of limited duration, which may explain why the healing following infarct is incomplete.

Prior to the present study, in order to validate our labeling technique to assure that this blue color by X-gal stain was not a false positive stain of injured myocardium per se, several hearts that had been coronary ligated, but never exposed to a previous injection of labeled MSCs were stained with X-gal solution. All of these hearts failed to show the presence of blue discoloration. In other words, only infarcted hearts with prior intravenous administration of labeled MSCs stained blue, as shown in Figs. 2 and 3.

Targeting of MSCs to the infarct site within the myocardium is also of interest. Chemotactic or other localizing factors may facilitate and guide the migration of MSCs. The trapping of the circulating MSCs by the endothelium of microvasculature in the infract and border zone of the myocardium may play important roles, but this has to be confirmed. The ability of MSCs to exit from vascular space into the tissue had been observed previously [2]. These cells then can undergo in situ differentiation, presumably under the influence of signals from the microenvironment. Although these cells can not fully reverse the damages incurred by the infarction, they may contribute to the healing process by participating in angiogenesis [14] to improve collateral circulation, to promote infarct scar maturation [6], to reduce scar expansion and rupture, as well as to modulate the post-infarct ventricular remodeling to preserve cardiac function [15].

The main limitation associated with our experimental design is the lack of precise quantitative data available in this study. Usually when bone marrow transplantation is performed, it is preceded by marrow ablation by radiation or chemotherapy, in order to create ‘space’ in the marrow for the transplanted cells to reside. We intentionally avoided marrow ablation procedure in order to prevent the collateral injury to the myocardium, which by itself may attract MSCs from the marrow, thus confounding our results. Consequently after the injection of $5 \times 10^6$ labeled MSCs, only 1–2% of the marrow cells were occupied by these labeled cells, so that the labeled cells we found in the heart may represent only 1–2% of total MSCs actually recruited to the myocardial infarct site. The quantity of cells mobilized may also be affected by the magnitude of the original insult, namely the infarct size, which was not examined in this study.

However, by creating and using an animal model, which has labeled MSCs residing in the bone marrow, we were able to demonstrate, both in gross and in microscopic specimens, a new dimension of the pathophysiology of myocardial infarction, which may be augmented by the therapeutic administration of exogenous MSCs. Such a ‘cellular cardiomyoplasty’ approach had indeed been reported to enhance recovery and improve cardiac function in animal models of myocardial infarction by a number of investigators [4,16].

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References


