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Marrow Stromal Cells Differentiate into Vasculature after Allogeneic Transplantation into Ischemic Myocardium

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Abstract

Background—Marrow stromal cells (MSCs) are reportedly able to improve ventricular function after MI through the paracrine effect or regenerating myocytes. However, the evidence to prove that is scant. In this animal study, we employed MSCs isolated from transgenic pigs designed to express enhanced green fluorescent proteins as the donor to study the fate of the cells after allogeneic transplantation.

Methods—Green MSCs prepared from transgenic pigs were allogeneically transplanted into chronic ischemic myocardium of eight Yorkshire pigs by direct intramyocardial injection (total 1.2×10^8 cells in 2.5 ml of saline, with 25 injection sites). Cohorts of two animals were sacrificed at 1, 2, 4, 6 wks and 3 months after injection to study the fate of the injected cells.

Results—Allogeneic injection of the green MSCs is safe, no observable side effects or signs of graft versus host disease were observed. By dapi counterstained frozen sections, the green cells were found migrating from the injected area into deeper layers of myocardium over the course of 1 to 6 weeks. By immunofluorescent staining, the green cells were associated with smooth muscle actin or vWF positive cells, suggesting that the transplanted cells were contributing to the formation of new vessels. We found no evidence that these cells were associated with the new generation of cardiac myocytes. Three months after injection, clusters of MSCs still can be found in the middle layer of ischemic myocardium, however, no unlimited cell growth was found.

Conclusions—Allogeneic transplantation of green MSCs can be safely used to elucidate the mechanisms of cell-based therapy. The benefits of this therapy appear mainly due to the angiogenesis not the regeneration of cardiac myocytes.

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Introduction

Adult marrow stromal cells (MSCs),¹ or previously referred to as “mesenchymal stem cells”,² have been used broadly both experimentally or clinically in myocardial ischemia or infarction to improve the ventricular function or presumably regenerate damaged myocytes.³⁻¹⁰ Most investigators believe that the functional improvement observed was due to the paracrine effect of the release of angiogenic factors by the transplanted MSCs.¹¹⁻¹³ The possibility that the MSCs lead to myocyte regeneration, or cell fusion, is still largely unproven. The major reason for this dilemma is that it is difficult to find a permanent marker to label the cells for tracking after transplantation and characterizing their fate. In this animal study, we chose to use recently developed transgenic pigs that express enhanced green fluorescent proteins¹⁴ as MSC donors to study the destiny of the cells after allogeneic transplantation.

Material and Methods

Animals

Two transgenic pigs, expressing enhanced green fluorescent proteins (EGFP) in all cell or tissue types, were obtained from the National Swine Resource and Research Center and served as green marrow stromal cell donors. The green pigs were developed by somatic cell nuclear transfer with porcine somatic cells transfected with EGFP driven by the chicken β -actin/rabbit β -globin hybrid promoter¹⁴. Ten Yorkshire domestic pigs, initially weighing 15-20 kg were used as green cell recipients for this study. All animals were housed one per cage and allowed free access to water and commercial pig food. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, and the investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996, Washington, D.C.).

Green MSCs preparation and culture

Using aseptic technique, bone marrow was aspirated from either the iliac crest or tibia of the two transgenic pigs into a syringe containing preservative free heparin. Peripheral RBCs were separated by gradient centrifugation using lymphocyte separation liquid. The middle layered cells were collected and divided into two populations: one population was cultured in DMEM with 10% FBS and 1X pen/strep in a density of $10^6/\text{cm}^2$ at 37°C with 5% CO₂ in T-75 culture flasks without coating. Three days later the non-attached floating portion of the mononuclear cells were collected and centrifuged and re-suspended in EGM2 medium. The attached colonies were then cultured with DMED and 10% FBS. When the attached cells reached confluence they were split and expanded for 2-4 passages. The second population of cells were cultured in growth factor enriched culture medium (EGM2) with 5% FBS. At three days, the non-attached floating portion of these cells were treated the same way as described above. Passage 4 cells were used for transplantation. In order to get enough cells for all of the experiments, the bone marrow aspiration procedure was repeated four times with alternation of each side of the iliac crest or tibia at three week intervals.

Flow cytometry analysis of cell surface markers

Cells from the two different culture conditions were seeded in a 10-cm dish and cultured to 80% confluence, then dispersed by PBS supplemented with 2 mmol/L EDTA and 1% bovine serum albumin. After being washed, the re-suspended cells are first incubated with Fc blocker at 4°C for 15 min, and then incubated with various specific antibodies labeled with phycoerythrin or FITC at the concentration of 2 µg/ml for 30 min. The mouse monoclonal anti-human antibodies (which cross react with the pig) of CD34, CD31, CD90, CD117(C-kit) and CD44 obtained from BD Biosciences (San Jose, CA), CXCR4 was from eBioscience (San Diego, CA), mouse anti-porcine antibodies of CD54, CD45, obtained from Serotec, (Raleigh, NC), rat anti-pig CD144 antibody was obtained from Antigenix America (Huntington Station, NY). After two washes, the cell surface markers were analyzed by FACSsan and CellQuest software (Becton-Dickinson FACScalibur).

Chronic Ischemia Model and green MSCs transplantation

Ten Yorkshire pigs, as the recipient of the green MSCs, were anesthetized and underwent a left-sided thoracotomy. The pericardial sac was partially opened to facilitate dissection and visualization of the proximal left circumflex (LCX) as it branches from the left main coronary artery. Then a titanium encased ameroid constrictor (2.5 mm in diameter) was placed around the proximal LCX. The pericardial sac was then closed to minimize adhesion formation. The ameroid constrictor gradually totally occludes the LCX over a period of 2-3 weeks resulting in a region of myocardial ischemia of the left ventricle. To help prevent arrhythmias, all animals were given amiodarone preoperatively beginning 5-7 days prior to the second surgery (200 mg b.i.d per os) which was continued until harvest. Four weeks later after ameroid placement, a second left thoracotomy was performed on each animal, the circumflex territory (ischemic zone) exposed and injected with ex-vivo expanded green MSCs from the two transgenic pigs (1.2×10^8) in 2.5 ml of saline intramyocardially with a 25 gauge needle (with an injection depth of 5 mm) throughout the ischemic zone (25 injection sites, 100 µl in each site). Two of these animals were sacrificed at one, two, four, six weeks and three months after injection to study the fate of the injected cells. At those time points, the hearts were harvested and sectioned. Cell differentiation was determined by immunohistochemistry staining with anti-vW factor antibody, smooth muscle actin and desmin.

Histological and immunohistochemistry analysis

After euthanization, ischemic (LCX territory) myocardium, site of the green cell transplantation, was cut into 5 × 5 mm-thick pieces, either collected in cassettes and fixed with 10% buffered formalin for paraffin embedding, or in O.C.T. for frozen sections with no fixation. Blood, lung, liver, kidney and spleen samples were also collected for green cell tracking. Paraffin embedded sections were stained with H&E and Masson trichrome for morphological analysis. The immunohistochemical or immunofluorescent staining was performed using rabbit polyclonal antibody against human vWF to detect vascular endothelial cells and mouse monoclonal antibody against human smooth muscle actin to detect vascular smooth muscle cells, and mouse monoclonal antibody against human desmin to detect myocytes. All above mentioned antibodies were purchased from Dako North

America (Carpinteria, CA). The incubations of primary antibodies were followed by detections of FITC conjugated anti-mouse IgG or Rhodamine conjugated anti-rabbit IgG and the nuclei were labeled with DAPI. CD163 was used to detect inflammatory cells.

Results

Cells

The MSCs prepared and cultured from the transgenic pigs showed similar morphologic characteristics as we have previously observed in regular Yorkshire pigs: at early passage they were round, irregular and spindle shaped cells forming different colonies with DMEM supplemented with 10% FBS. The growth speed of the cultured bone marrow cells was much slower from passage 0 to passage 2, but dramatically increased starting from passage 3, and demonstrated uniformed fibroblast-like morphology (Figure 1 c, d). The cells cultured with growth factor enriched EGM2 medium grew faster and were smaller in size than the cells in DMEM, but demonstrated similar spindle shape morphology (Figure 1 a, b). The green MSCs were ex-vivo expanded for four passages before use for transplantation. All cells exhibited strong green fluorescence under microscope starting at passage 0 to passage 4.

FACS analysis at passage 4 revealed that the green MSCs were strongly positive for cell surface markers CD44 and CD 90, but CXCR4, CD34, C-kit, CD144, CD54, CD45, CD31, were all negative. These results indicate that bone marrow-derived stem cells cultured in the aforementioned two conditions are marrow stromal cells not endothelial progenitor cells.

Animals

All animals that received allogeneic transplantation of green MSCs showed no signs of graft-versus-host disease. One animal died of surgical complications and infection at 10 days after cell injection.

Histologic Analysis

Time course analysis—Frozen sections with dapi counterstaining was used to track the transplanted green MSCs. At one week after injection clusters of injected cells, small and round shaped, with strong green fluorescence were found just under the epicardial surface (Figure 2 a, b). At two weeks, green MSCs were found migrating into the deeper intramyocardial layer. These cells stained strongly with hematoxylin, and were round shaped in morphology (Figure 2 c, d). After 4 weeks green MSCs were found in the ischemic area in line with interstitial connective tissues (Figure 2 e). At six weeks green MSCs can still be seen in the middle layer of myocardium. In addition to their depth of penetration the cells appeared less aggregate and more dispersed following diffuse migration.

Differentiation analysis at the six week time point—By immunofluorescent staining with smooth muscle actin, the green MSCs were found associated with small vessels in the fibrotic foci within the ischemic zone (Figure 3 a, b). Using immunofluorescent vWF staining, green MSCs were also found associated with vWF positive cells (Figure 3 c-f). These results suggested that the transplanted MSCs were more likely to differentiate into

vasculature or become incorporated into small vessels. By studying the H & E staining sections we also found cross sections of clusters of small cellular tubes in the ischemic myocardium with MSCs (Figure 4 a), which stained positively for smooth muscle actin in the wall and a few positive vWF cells in the inner layer of the tubes (Figure 4 b). This evidence suggests that these tubes are newly formed small vessels. Six weeks following injection, green MSCs were not found associated with myocytes when co-stained with desmin, a myocyte marker (Figure 4 c, d). In areas with a preponderance of green cells, we found no signs of cardiomyocyte differentiation morphologically and they did not stain for desmin, while in the normal myocardial area the myocytes were strongly positive for desmin (Figure e, f).

Long-term observations—At three months after injection with H&E staining clusters of transplanted MSCs can be found in the middle area of the ischemic myocardium, showing the same characteristics found in 6 weeks (Figure 5). With higher magnification, cells inside the clusters were found to be surrounded by pink-colored spindle-shaped tissues, which proved to be collagen fibers (Masson Trichrome staining), not myocytes (figure 5). This data confirmed that the MSCs transplanted through direct epicardial injection survived and stayed inside the ischemic area for at least three months without unlimited outgrowth, suggesting a safe clinical application.

Green cells distribution and inflammatory cell infiltration—The green cells were not found in the lung, kidney, spleen and liver at 2, 4 and 6 weeks following transplantation. Very few green cells were found in the lung and blood one week after injection. Other than a few positive CD163 staining cells found in the fibrotic area no graft-versus-host rejective sign was found at 6 weeks after allogeneic cell transplantation.

Comment

We previously found that in the swine model of chronic myocardial ischemia, the direct injection of autologous marrow stromal cells improves ventricular wall motion both globally and regionally.¹⁵ The mechanism that contributes to the improvement still has to be delineated. To track their fate, migration, and differentiation after transplantation, the cells need to be labeled with dye, BrdU, or GFP transfection through retro or lenti-virus vectors. Not all these methods are perfect due to the cross contamination by dye, short duration of marker, transfection efficiency, or a limited period of expression. Recently, transgenic pigs expressing enhanced green fluorescent proteins were developed by Whitworth and colleagues¹⁴ and commercially available through the National Swine Resource and Research Center. We successfully isolated and ex vivo expanded the bone marrow stromal cells from these transgenic pigs. These brightly fluorescent green MSCs have all the characteristics of our previously developed swine MSCs. In this study, we sought to use the green MSCs to determine the fate of the adult marrow stromal cells following direct epicardial injection.

The convenient way to perform cell therapy is to allogeneically transplant cells to recipients, or use a developed cell line for all recipients, since theoretically such stem cell lines would lack MHC1 and should not incite rejection. However, there is no solid evidence to prove that is always the case. The clinical trials of cell-based therapy in cardiovascular diseases for the

past 10 years were therefore limited to autologous use of the patients' own bone marrow cells.¹⁶⁻²⁴ Similarly, in order to avoid possible rejection related issues, we also chose to use autologous cells in our previous study. However, Hare and colleagues²⁵ had just reported a clinical trial using a human MSCs cell line developed by Osiris Therapeutics Inc. In this study, hMSCs, were administered intravenously to patients with acute myocardial infarction. They found that the allogeneic stem cell therapy was safe, no GvHD was observed, and symptoms and LV function were improved in hMSCs treated patients compared with placebo injected patients. The study suggested the potential usage of allogeneic cell therapy in the future.

In our present study the allogeneic transplantation of MSCs did not cause any GvHD response and no neoplasm has been found up to three months following injection.

The preparation and handling of the green MSCs are almost the same as our previously described experiences handling the MSCs in Yorkshire strain except that green cells are more susceptible to temperature variations. They tend to be less adherent when cultured in cold media. We also found that when we subculture them accutase is much better than trypsin-EDTA. In preparing the cells used in this study, we also chose to use two standard culturing methods. We found that cells cultured by either method shared the same characteristics of morphology and cell surface markers except the growth rate was faster in EGM2 than in DMEM. In the time course study, we injected cells from both culture conditions separately and saw no difference in their differentiation.

Summary

In this study, we found that the allogenic green MSCs survived in the myocardial environment, migrated into the deeper layers of the ischemic myocardium and differentiated into vasculature cells, smooth muscle or endothelial cells. We cannot exclude the possibility that those new regenerated cellular tubes may also be the result of the paracrine effect of the injected cells causing recruitment from other sources. However, the histological findings in our study strongly suggest that transplanted cells actively formed or were incorporated into these newly formed vessels. Also, if it is primarily a paracrine effect, it would more likely lead to diffuse angiogenesis, not just near the injected areas. In our study we did not see any evidence that the injected MSC differentiated into functional or morphologically analogous cardiac myocytes up to three months following direct injection.

The beauty of this current study is to use maturely green fluorescent labeled cells directly injected into chronically ischemic myocardium which better mimics the clinical scenario and traces the fate of the injected cells. Our data further confirmed that at various time points after injection the cells survived and produced evidence of angiogenesis. However, there was no myocyte regeneration incited by MSCs transplantation.

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Figure 1.

A) Phase contrast image of green MSCs cultured with EGM2 medium, passage 1 at day 3 (x200); B) Fluorescent image of A; C) Phase contrast image of green MSCs cultured with DMEM medium, passage 1 at day 5 (x200); D) Fluorescent image of C.

Figure 2.

Time course analysis for the tracking of transplanted allogeneic green MSCs using frozen section with dapi counterstaining. A & B: One week following injection, green MSCs were found around the epicardial injection site (x200); C: At two weeks following injection, green MSCs were found migrating into deeper myocardial layers (x200); D: Same section without fluorescence; E: After 4 wks, green MSCs were found in the ischemic area in line with interstitial connective tissues; F: At six weeks, green MSCs were found in the middle layer of myocardium.

Figure 3.

At six weeks following injection, green MSCs were found associated with small vessels in the ischemic area. A: H & E staining of the ischemic area; B: Smooth muscle actin staining (red); C-F: vWF staining (red).

Figure 4.

A: At six weeks a cross section of clusters of small cellular tubes were found in the ischemic myocardium with MSCs transplantation, believed to be newly formed small capillaries (H&E); B: Immunofluorescent staining with smooth muscle actin (long arrow), and vWF (short arrow); C & D: Six weeks following injection, green cells were found not associated with myocytes which stained red with desmin; E: Injected MSCs survived, however, showed no sign of cardiomyocyte differentiation morphologically and negatively stained for desmin, a marker for myocytes; F: Positive staining of desmin in normal pig myocardium.

Figure 5.

Three months after injection, groups of dark stained cells were found in the center of the myocardium of the injected sites. A) Full-length of myocardium with cell injection: Black arrows indicate epimyocardial area, white arrows indicate injected cells (x12.5); B) Higher magnification of defined area of A (x100); C) Higher magnification of B (x400); D) Masson Trichrome (Collagen) staining of C.