Autologous Transplantation of Bone Marrow Cells Improves Damaged Heart Function

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Background—Autologous bone marrow cells (BMCs) transplanted into ventricular scar tissue may differentiate into cardiomyocytes and restore myocardial function. This study evaluated cardiomyogenic differentiation of BMCs, their survival in myocardial scar tissue, and the effect of the implanted cells on heart function.

Methods and Results—In vitro studies: BMCs from adult rats were cultured in cell culture medium (control) and medium with 5-azacytidine (5-aza, 10 μmol/L), TGFβ1 (10 ng/mL), or insulin (1 nmol/L) (n=6, each group). Only BMCs cultured with 5-aza formed myotubules which stained positively for troponin I and myosin heavy chain. In vivo studies: a cryoinjury-derived scar was formed in the left ventricular free wall. At 3 weeks after injury, fresh BMCs (n=9), cultured BMCs (n=9), 5-aza–induced BMCs (n=12), and medium (control, n=12) were autologously transplanted into the scar. Heart function was measured at 8 weeks after myocardial injury. Cardiac-like muscle cells which stained positively for myosin heavy chain and troponin I were observed in the scar tissue of the 3 groups of BMC transplanted hearts. Only the 5-aza–treated BMC transplanted hearts had systolic and developed pressures which were higher (P<0.05) than that of the control hearts. All transplanted BMCs induced angiogenesis in the scar.

Conclusions—Transplantation of BMCs induced angiogenesis. BMCs cultured with 5-aza differentiated into cardiac-like muscle cells in culture and in vivo in ventricular scar tissue and improved myocardial function. (Circulation. 1999;100[ suppl II]:II-247–II-256.)

Key Words: cells ■ myocardial infarction ■ transplantation ■ angiogenesis ■ myocardial contraction

Fetal cardiomyocytes transplanted into myocardial scar tissue improved heart function.1,2 However, the transplanted allogenic cells survived for only a short time in the recipient heart because of immunorejection.3 Autologous cell transplantation would be ideal. Cultured skeletal myoblasts have been successfully isolated, cultured, and transplanted into injured and normal myocardium of the same animal.4,5 Several researchers believe that skeletal myoblasts can differentiate into cells resembling cardiac muscle cells when injected into the myocardium. Approximately 4% to 8% of mammalian skeletal muscle cells are skeletal myoblasts.6 Because this percentage decreases with the age of the mammal, a large quantity of muscle will be required to obtain sufficient numbers of cultured skeletal myoblasts to improve heart function. Skeletal muscle cells do not form gap junctions in vivo, making it unlikely that the transplanted cells will contract synchronously with the host myocardium.

Bone marrow has multipotential progenitor cells which can differentiate into muscle, cartilage, bone, fat, and tendon.7 Chemicals have been reported that induce bone marrow cells (BMCs) into myogenic cells.7–9 Because BMCs are easily obtained, transplantation should be successful if the cells can be directed to differentiate into myogenic cells, ideally cardiomyocytes.

In this study, we induced cultured adult rat BMCs into myogenic cells which express cardiac muscle cell markers, troponin I, and myosin heavy chain. The induced cells were autotransplanted into myocardial scar tissue produced by cryoinjury. The transplanted cells formed cardiac-like cells in the scar and induced angiogenesis. Heart function improved in only the 5-aza–treated bone marrow transplants.

Methods

Animals

Adult Sprague-Dawley rats (Charles River Canada Inc, Quebec, Canada) were used. All procedures, approved by the Animal Care Committee of The Toronto Hospital, were performed according to the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care and Use of Laboratory Animals published by the National Institute of Health (NIH publication 85-23, revised 1985).

Preparation of BMCs

The rats were anesthetized with intramuscular administration of ketamine hydrochloride (22 mg/kg) followed by an intraperitoneal injection of sodium pentobarbital (30 mg/kg). Under general anesthesia, bone marrow was aspirated from the tibia with a syringe containing 1 mL heparin with an 18-gauge needle. The marrow cells
were transferred to a sterile tube and mixed with 10 mL culture medium (Iscove’s modified Dulbecco medium: IMDM with 10% fetal bovine serum, penicillin G [100U/mL] and streptomycin [100 µg/mL]). The tube was centrifuged at 2000 rpm for 5 minutes and the cell pellet was resuspended with 5 mL culture medium. To separate BMCs and red blood cells, the gradient centrifugation method described by Yablonka-Reuveni and Nameroff was used. The cell suspension was loaded on 20% to 60% gradient of Percoll. The cells were centrifuged at 14 000 rpm for 10 minutes. The top two thirds of total volume were transferred into a tube (a preliminary study showed that these layers contained most of the BMCs). The cells were centrifuged at 2 000 rpm for 10 minutes and then washed with PBS to remove the Percoll. This was repeated and the cell pellet was then resuspended in culture medium and used for in vitro and in vivo studies.

The cells were cultured in IMDM containing 10% fetal bovine serum and antibiotics, with 5-aza (0.1, 1, 5, 10, 20, and 100 µmol/L), insulin (1 nmol/L, n=6), or transforming growth factor (TGFβ1, 10 ng/mL, n=6). The cells were incubated with these chemicals for 24 hours and then washed with PBS. The medium was changed twice a week for 21 days. With the medium changes, almost all the hematopoietic stem cells were washed away.

Identification of Cardiac Contractile Proteins in the Cultured BMCs

The cultured cells were immunohistochemically stained for cardiac-specific troponin I and myosin heavy chain. Briefly, the cells were washed with PBS and treated with methanol at −20°C for 20 minutes. The dishes were washed with PBS 3 times. A monoclonal antibody against cardiac-specific troponin I (27 µg/mL) (Spectral Diagnostic) and a monoclonal antibody against myosin heavy chain (Biogenesis) were used. The cells were incubated with the antibodies at 37°C for 1 hour. To remove the unbound antibody, the culture dishes were gently shaken at room temperature and washed 3 times with PBS. A rabbit antibody conjugated with FITC against mouse IgG was added to the dishes. Incubation and rinsing with PBS were performed as described above for the first antibody. The cells were then photographed.

Myocardial Scar Generation

Under general anesthesia, adult rats were intubated, and positive-pressure ventilation (180 mL/min.) was maintained with room air supplemented with oxygen (2L/min) using a Harvard ventilator (model 683). The rat heart was exposed through a 2-cm left lateral thoracotomy. Cryoinjury was produced with a metal probe (8×10 mm in diameter) cooled to −190°C by immersion in liquid nitrogen and applied to the left ventricular free wall for 15 seconds. This procedure was repeated 5 times and then applied for a total of 10 times with each time lasting 1 minute. The muscle layer and skin incision were closed with 3-0 silk sutures. The rats were monitored for 4 hours postoperatively. Penlong XL, penicillin G benzathine (150 000 U/mL), and penicillin G procaine (150 000 U/mL) were given intramuscularly (0.4 mL per rat).

Cell Preparation for Transplantation

Three weeks after myocardial damage, the rats were randomly divided into 4 groups. Group 1 (n=9): BMCs freshly prepared, as described in Materials and Methods, were resuspended in IMDM and transplanted by injecting into the center of the scar tissue. Group 2 (n=9): BMCs were cultured for 7 days before transplantation. Group 3 (n=12): BMCs were cultured for a total 7 days. 5-aza (10 µmol/L) was added on the third day and incubated with cells for 24 hours. Group 4 (n=12): IMDM was injected as the control.

The cultured cells were dissociated from the culture dishes with 0.05% trypsin (Gibco BRL, Grand Island, NY), neutralized with culture medium, and collected by centrifugation at 2000 rpm for 5 minutes at room temperature. The cells were suspended in IMDM at concentration of 10⁶ cells in 50 µL for transplantation.

Cell Transplantation

The rat heart was exposed through a midline sternotomy under general anesthesia; 50 µL of BMC suspension containing 10⁶ cells

Figure 1. Bone marrow cells in culture (magnification×200). The majority of the cultured bone marrow cells were spindle-like mesenchymal stem cells after 7 days of culturing and purification.
Figure 2. A, Bone marrow cells cultured with 10 μL 5-aza (magnification ×200): bone marrow cells were cultured for a total of 10 days with 5-aza added to the medium only on the third day. Only the BMCs cultured with 5-aza formed a network of myotubules. Occasional adipocytes were present. B, Cultured bone marrow cells immunohistochemically stained for troponin I (magnification ×400): cultured bone marrow cells were treated with 10 μL 5-aza for 24 hours on day 3 of a 10-day culture. Myotubular cells stained positively for troponin I but not the other cell types.
were injected using a tuberculin syringe into the center of the left ventricular free wall scar tissue of each animal in transplant groups 1, 2 and 3. Fifty microliters of IMDM were injected into the center of the scar tissue of the control animals (group 4). The chest was closed with 3-0 silk sutures. Antibiotics and analgesics were given as previously described.

Heart Function Measurements
Five weeks after transplantation, the rats were anesthetized with ketamine and pentobarbital as previously described.2 A midline sternotomy was performed, the heart was removed, and the animals were euthanized by exsanguination. Heart function of the 4 groups was measured using a Langendorff apparatus with filtered Krebs-Henseleit buffer (in mmol/L: NaCl, 118; KCl, 4.7; KH₂PO₄, 1.2; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 25; and glucose, 11; pH 7.4) at the pressure of 100 mm Hg equilibrated with 5% CO₂ and 95% O₂). A latex balloon was passed into the left ventricle through the mitral valve and connected to a pressure transducer (model p10EZ, Viggo-Spectramed), a transducer amplifier, and a differentiator amplifier (model 11-G4113-01, Gould Instrument System Inc). After 30-minute stabilization, the coronary flow of the heart was measured in triplicate by timed collection in the empty beating state without pacing. The balloon size was increased by the addition of water in 20-μL increments from 40 μL until the left ventricular end-diastolic pressure reached 30 mm Hg. The systolic and diastolic pressures were recorded at each balloon volume and developed pressure was calculated. The heart was weighed and its size was measured by water displacement.

Planimetry
The scar size of left ventricular free wall was measured by the techniques of Pfeffer and associates11 and Jugdutt and Khan.12 Briefly, the hearts were fixed in distention (30 mm Hg) with 10% neutralized formalin and then cut into slices 3 mm thick. For each section, the outer and inner lines of the left ventricle were traced onto a transparency and quantified using computed planimetry (Jandel Scientific Sigma-Scan).

Histological Studies
Tissue samples (0.5cm³) at the transplantation site were collected at 5 weeks after transplantation and fixed in neutralized 10% formaldehyde for histological study. The samples were embedded and cut to yield 10-μm thick sections, which were stained with hematoxylin and eosin as described in the manufacturer’s specifications (Sigma Chemical Co).

Identification of Transplanted BMCs in the Scar
Under general anesthesia, 4 rats were scarred and 2 weeks later bone marrow was aspirated. The BMCs were cultured and induced with 5-aza as described above. To identify the transplanted cells in the scar tissue, the cells were labeled with bromodeoxyuridine (BrdU, Sigma). Briefly, 10 μL of BrdU solution (BrdU, 50 mg; dimethyl sulfoxide, 0.8 mL; water, 1.2 mL) was added into each culture dish on the sixth day of culture and incubated with the cells for 24 hours. Labeling efficiency was ~75%. The labeled cells were transplanted into the scar at 3 weeks after myocardial injury, and samples were collected at 5 weeks after transplantation as previously described. Monoclonal antibodies against BrdU were used to localize the transplanted bone marrow cells.13 Briefly, samples were serially dehydrated with 100%, 95%, and 70% ethanol after deparaffinization with toluene. Endogenous peroxidase in the sample was blocked using 3% hydrogen peroxide for 10 minutes at room temperature. The sample was treated with pepsin for 5 minutes at 42°C and 2N HCl for 30 minutes at room temperature. After rinsing with PBS 3 times, the sample was incubated with antibodies against BrdU in a moist chamber for 16 hours at room temperature. Negative control samples were incubated in PBS (without the primary antibodies) under the same conditions. The test and control samples were rinsed with PBS 3 times (15 minutes each) and then incubated with goat anti-rabbit immunoglobulin G conjugated with peroxidase at 37°C.
for 45 minutes. The samples were washed 3 times (15 minutes each) with PBS and then immersed in diaminobenzidine H₂O₂ (2 mg/mL diaminobenzidine, 0.03% H₂O₂ in 0.02 mL/L phosphate buffer) solution for 15 minutes. After washing with PBS, the samples were coverslipped and photographed.

Measurement of Capillary Density in the Scar

The number of capillary vessels was counted in the scar tissue of all groups, using a light microscope at a ×400 magnification. Five high-power fields in each scar were randomly selected, and
The numbers of cells in the culture in which 5-aza was added on first and second day were much smaller than that of the cells with 5-aza added on the third day.

The nuclei of the cells induced by 5-aza were labeled with BrdU for 24 hours pretransplantation; 75.3±4.3% of the cultured cells stained positively. The labeled cells were transplanted into the myocardial scar tissue. At 5 weeks posttransplantation, BrdU-stained cells were observed at the transplanted area (Figures 3). The BrdU-stained cells were muscle-like cells and stained positively for troponin I (Figure 4). Muscle-like cells were formed in the scar tissue in all bone marrow transplanted animals but not in the control scars, which were homogeneous in appearance and did not contain any host cardiomyocytes. Transplants of fresh bone marrow cells, cultured bone marrow cells, and 5-azacytidine–treated bone marrow transplants stained positively for troponin I.

The transplanted BMCs stimulated angiogenesis. The number of capillaries of the BMC transplanted groups (fresh: 6.29±0.58, BMCs: 5.93±0.33, BMCs+5aza: 5.74±0.57 vessels/0.2 mm2) was larger (P<0.05) than that of the control group (2.12±0.38 vessels/0.2 mm2) (Figure 5). Some capillary walls were composed of BrdU-positive endothelial cells (Figure 6). No lymphocyte infiltration and immunorejection were evident. Cartilage, bone, and fat did not form in the transplanted area. No tumor-like cells were seen.

Morphological studies showed that transmural scar area of the 5-aza group was smaller (P<0.05) than the other groups (Figure 7), and scar thickness of the 5-aza group was larger (P<0.05) than the other groups (Figure 8). Left ventricular volume/body weight of control group was significantly larger than those of BMC transplantation groups (Figure 9). Figure 10 shows the results of the left ventricular function studies of the transplanted and control hearts. Peak systolic and developed pressure of 5-aza–treated BMC transplants were better (P<0.05) than those of the other groups. No difference in function was found among the fresh bone marrow, cultured bone marrow, and control transplants.

Results

Two major types of cells, mesenchymal stem cells (MSCs) and hematopoietic stem cells, were prepared by Percoll gradient centrifugation technique. The MSCs were spindle-shaped, attached to the culture dish tightly, and proliferated in the culture medium (Figure 1). The hematopoietic stem cells were round, did not attach to the culture dish, and were washed away with the culture medium changes. On day 7, almost all the cells in the culture dish were spindle-shaped MSCs. When the cells were cultured with only 5-aza, they linked, formed myotubules, and had multinuclei on the tenth day of culture (Figure 2A). The cells stained positively for cardiac specific troponin I (Figure 2B) and myosin heavy chain on the 21st day. Purified BMCs, cultured with TGFβ1 and insulin or the medium, did not form myotubules and stained negatively for troponin I and myosin heavy chain.

The optimal conditions for BMCs to differentiate into muscle cells were evaluated by incubating BMCs with 0.1, 1, 5, 10, 20, and 100 μmol/L 5-aza. BMCs incubated with 5-aza at 20 and 100 μmol/L appeared abnormal. More than 50% of the cells necrosed. Although damaged BMCs were also observed in the culture with 5 and 10 μmol/L 5-aza for 1 day, myotubule formation was present. The number of myotubules in the 10 μmol/L 5-aza was visually greater than in the 5 μmol/L group. Cells cultured with <5 μmol/L 5-aza were morphologically similar to the control cells. BMCs were incubated with 10 μmol/L 5-aza on the first, second, and third day to optimize the time for 5-aza to stimulate BMC differentiation. The numbers of cells in the culture in which

Data Analysis

Data are expressed as mean±SE. Statistical Analysis System software (SAS Institute) was used for all analyses. Comparisons of continuous variables between >2 groups were performed by a 1-way ANOVA. If the F distribution was significant, a Scheffe’s test was used to specify differences between groups. This analysis was performed at each balloon volume. The critical α-level for these analyses was set at P<0.05.

Discussion

Cell transplantation may be one alternative treatment for heart failure in the future. The best cell to be transplanted and the source of the cells to be isolated are important considerations. Xenotypic cells may be used if cell surface markers can be altered to reduce immunorejection. Allogeneic cells have been successfully transplanted by several groups. The transplanted cells survived in normal myocardium and scar tissue with the use of cyclosporine A and improved heart function. Unfortunately, a long-term study demonstrated that allogeneic cells were immunologically rejected by 24 weeks after transplantation despite cyclosporin A therapy. Because of the disadvantages of immunorejection, autologous cell transplantation would be an ideal technique. However, cardiac and skeletal muscle biopsies may not yield sufficient cell numbers to repair the myocardium.

Many researchers have induced cultured, nonmyogenic cells to differentiate into myogenic cells using chemicals. Cardiac fibroblasts were converted into cardiomyogenic cells with TGFβ3. Insulin induced L6 and C2 cell lines to differentiate into myogenic cells. Bone marrow is routinely aspirated clinically and contains multipotential progenitor
cells which can differentiate into muscle cells. In our study, although we used both TGFβ1 and insulin at a variety of concentrations to induce the differentiation of BMCs, the cells did not differentiate into myogenic cells containing contractile proteins, troponin I, and myosin heavy chain. Wakitani et al demonstrated that 5-aza in culture medium induced bone marrow mesenchymal stem cells to differentiate into myogenic cells. In agreement with this finding, we
showed myotubule-like cells when 5-aza was added to the culture media; these cells were not present in the control group. The cells stained positively for troponin I and myosin heavy chain. The precise mechanism by which 5-aza induced BMCs to differentiate into muscle cells is unknown. In studies on myogenic differentiation of the mouse embryonic cell line, C3H10T1/2 with 5-aza, Konieczny et al proposed that these cells contain a myogenic determination locus in a methylated state with a transcriptionally inactive phase, which becomes demethylated and transcriptionally active with 5-aza causing the cells to differentiate into myogenic cells. Our data indicated that BMC treated with 10 μmol/L 5-aza for 24 hours provided the optimal concentration for BMC differentiation into myogenic cells with cardiomyocyte-like characteristics. The induction of 5-aza was performed on the fourth day of culture because osteogenic factor alkaline phosphatase synthesis begins on day 4. We thought this was important to minimize the possibility of bone formation in the transplantation.

The cardiac milieu had an important effect on the myogenesis. The BMCs were cultured for 7 days before transplantation so that the immature bone marrow cells would differentiate in vivo in the scar and not in vitro. Although adipocytes were present in the BMCs cultured with 5-aza, fat tissue was not found in the transplanted area. In vitro, the bone marrow cells did not differentiate into myogenic cells without 5-aza. In contrast, fresh bone marrow cells and cultured bone marrow cells with or without 5-aza differentiated into myogenic-like cells in the transplant. In vivo myogenic factors, in addition to 5-aza, were important in the differentiation of the immature BMCs to cardiomyocyte-like cells.

An understanding of the limitations of the in vivo study is important to better understand the significance of the results. The cryonecrosis model of a myocardial infarction has advantages and disadvantages compared with the coronary ligation model in the rat. The cryonecrosis scar was a homogeneous transmural scar located at a fixed location and of a fixed size. Injection of the cells into the center of the scar facilitated identification of the transplant cells. Function measurements showed less between animal variability because the injury in our model involved only the left ventricular free wall and was of constant size. In a coronary artery ligation model, the size and location of scar was variable. Transplant identification would be more difficult and functional results would be more variable. The major advantage of the ligation model is that it is clinically relevant whereas the cryonecrosis model is not. An arbitrary number of BMCs was injected into the center of the myocardial scar. The optimal number of cells to maximize myocardial function was not determined. Because the scar was subjected to systolic pressures, cell leakage could be a problem. With a coronary ligation model, more improvement in contractile function would be expected. The transplant-induced angiogenesis should restore contractile function to hibernating host cardiomyocytes. If the transplant had been in contact with contract-
ing host cardiomyocytes, in vivo myogenic factors from the host cardiomyocytes might differentiate the transplanted BMCs into mature contracting cardiomyocytes. Makino et al showed that 5-aza–treated murine-immortalized bone marrow stromal cells became contracting cardiomyocytes after 2 weeks in culture. In our studies, we treated the cultured rat BMCs differently. More research is required to optimize the treatment of the BMCs with 5-aza.

Only the transplants with BMC induced by 5-aza inhibited the ventricular scar from thinning and expanding minimized left ventricular chamber dilatation and improved myocardial function compared with the control hearts. There are many possible explanations. Although myogenic cells from BMCs have in vitro contractile function, it is unlikely that they contributed significant in vivo contractile function. The transplanted cells were not in contact with the host cardiomyocytes and would not have beat synchronously with the host myocardium. It is likely that the 5-aza–derived cardiomyocyte-like cells were more elastic than the untreated cardiomyocyte-like cells and prevented scar expansion and ventricular chamber dilatation. On the basis of our in vitro findings, we speculate that the 5-aza–treated BMCs contained more contractile structures that provided the cells with more elasticity than the untreated BMCs. Preventing overstretching of the host ventricular cardiomyocytes will permit their normal contractile function (Frank-Starling Law). These findings are in agreement with the clinical results of surgical reconstruction of the left ventricle with an aneurysm. Reduction of the chamber size improved heart performance.

All bone marrow cell transplantation groups induced angiogenesis in the scar. Bone marrow has endothelial progenitor cells that could contribute to new capillary formation in the acute ischemic hindlimb model. Because few of the capillary wall endothelial cells were labeled with BrdU, the majority of the endothelial cells must have originated from the host myocardium. On the basis of other studies in our laboratory, we believe that the increase in capillary density in the scar would also be mirrored by increased 57Co-labeled microspheres uptake. Marat and Doulet reported bone marrow cell transplantation accelerates revascularization with aid of basic fibroblast growth factor in an acute ischemic dog heart model. The neovascularization may have been important in the long-term survival of the transplanted cells in the scar. Because all the BMC transplants showed similar increases in capillary density and only the 5-aza–derived BMC transplants improved myocardial function, the neovascularization by itself did not contribute to the improved function. We have also shown in a cryoinjury model that endothelial cell transplantation induced angiogenesis but did not improve heart function. In a coronary ligation model, neovascularization should improve contractile function by improving blood flow to hibernating cardiomyocytes.

**Figure 10.** Systolic, diastolic, and developed pressures of the fresh BMCs, cultured BMCs, 5-aza–treated BMCs, and control hearts with increasing balloon volumes. The systolic and developed pressures of the 5-aza–treated BMC transplant hearts were better \((P<0.05)\) than those of the other hearts. \(n=9\) for fresh and cultured BMC hearts; \(n=12\) for 5-aza-treated and control hearts.

**Conclusion**

BMCs should be considered as an alternative transplant cell source to repair the damaged myocardium. BMCs were induced to differentiate into cardiomyogenic cells with 5-aza. Transplantation of the 5-aza–treated BMCs into the scar of the failing heart improved contractile function and all transplanted BMCs induced angiogenesis.
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