Homing of peripherally injected bone marrow cells in the rat after experimental myocardial injury

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Background and Objectives. Significant progress has been achieved during the past 10 years in cell transplantation and recent research has focused on the possibility of improving ventricular function after myocardial infarction. Most studies in the field of cardiac tissue repair are performed by direct intramyocardial injection of cells of different origin. Since this approach requires a surgical intervention, in this study we investigated the feasibility of non-invasive administration of bone marrow mononuclear cells (BMMNCs) by assessing the fate of peripherally injected, purified, labeled cells in cryodamaged hearts.

Design and Methods. Ten donor and ten recipient inbred isogenic adult (4 weeks old) Fisher rats were used as models to mimic autologous transplantation. Myocardial damage was obtained in recipient rats by placing a frozen metal probe on the anterior left ventricular wall for 15 seconds (freeze-thaw injury technique). BMMNCs were purified and labeled with a red fluorescent cell dye. Seven days after the injury about $15–25\times10^6$ cells were infused through the femoral vein of recipient rats. Seven days after the infusion, the heart, lungs, liver, kidneys, spleen and thymus were harvested to track transplanted cells.

Results. Labeled cells were found only in the injured area of the heart and not in the normal tissue; a limited number of cells were also identified in the spleen of all the animals. Most of the labeled cells in the infarcted area were Thy-1^+ and some were $CD34^+$.

Interpretation and Conclusions. Our data suggest that peripherally injected BMMNCs can traffic through the circulation to the site of damage; we hypothesize that tissue injury leads to the priming of a cytokine cascade acting as chemoattractant for the infused cells.

Key words: bone marrow, cryoinduced myocardial damage, systemic injection.

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Recently it has been demonstrated that stem cells, particularly those from the bone marrow (BM), have the capacity to colonize different tissues, proliferate and transdifferentiate into cell lineages of the host organ.[1,2] In fact, it has been shown that there are pluripotent progenitor cells in the BM which can differentiate into muscle, cartilage, bone, fat and tendon.[3,4] Therefore, it has been proposed that somatic cell therapy protocols can be developed to treat a number of diseases, including cardiovascular ones. In this regard, several authors have recently investigated different strategies to renew myocardium after myocardial infarction, including direct transplantation of cardiomyocytes or skeletal myoblasts.[5,6] However, so far these approaches have invariably failed to reconstitute healthy myocardium and coronary vessels structurally and functionally integrated with the ventricular wall.[7] The injection of BM stromal cells, containing adult stem cells, into the contracting myocardium bordering an acute infarct, has been proven to be feasible,[8,9] as shown by the homing and transdifferentiation of these cells into cardiomyocytes. Most studies in this field were performed by direct injection of various cells into the myocardium. However, this approach requires a surgical intervention that may be associated with significant morbidity and mortality as well as with a limited grafting success rate. More recently the possibility of injecting BM stromal cells directly into the clamped aorta, with subsequent migration of the cells through the coronary arteries to the infarction scar, has been reported.[10] In this study we tested the hypothesis that tissue damage acts as a chemoattractant for stem cells by assessing the fate of peripherally injected, purified, labeled bone marrow mononuclear stem cells (BMMNCs) in rats with artificially injured cardiac muscle.

Design and Methods

Animals

In order to mimic autologous infusion of BMMNCs for future clinical application, we used an animal model, carrying out our experiments in 23 male adult inbred Fisher rats (Charles River Laboratories, Italy), weighing 200-250 g. Animals were housed and handled in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (1996 revision) and the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care.

Experimental injury

Cardiac injury was induced using the freeze-thaw technique in 13 recipient rats (10 subsequently treated animals and 3 controls). This technique, previously described in a model of myoblast transplantation for the repair of myocardial necrosis,[5] consists in positioning a precooled (liquid nitrogen, -190°C) aluminum rod (9 mm diameter) at the target location. The resulting injury is then produced by creating a negative thermic gradient from the tissue to the contiguous precooled instrument. In our study, the animals were placed under diethyl ether anesthesia and spontaneous respiration in the right lateral decubitus position. After immobilization, they underwent depilation and disinfection of the left thorax. The heart was then rapidly exposed aseptically via a left thoracotomy through the third or the fourth intercostal space; the myocardial damage was produced by placing the probe in direct contact with the anterior left ventricular wall for 15 sec (Figure 1). The exact position of the instrument was carefully fixed by using the right auricular appendage and the origin of the pulmonary trunk as anatomical landmarks. The cryodamaged area was macroscopically identified as a firm, white disk-shaped region of coagulation necrosis. After the injury, the heart was rapidly repositioned within the thoracic cavity and the chest closed with 5-0 vicryl sutures.

Echocardiography

The myocardial lesion was assessed in the acute setting (24 hours after the injury) by transthoracic echocardiography. The rats were examined, with the chest closed, under diethyl ether anesthesia, in the left lateral decubitus position, using an echocardiographic system (Kontron Sigma 44 HVD, Les Gatines, France) equipped with a 7.5 MHz mechanical probe (focus depth set at 3.0 cm, sectorial angle of 60°). In order to identify the phase of the cardiac cycle, 3 electrodes were attached to the animal's paws to obtain a simultaneous electrocardiographic tracing. Infarct size was estimated at end diastole from the short-axis echoes by observing the akinetic region in real time and measuring (planimetry) the percentage of the endocardial circumference of the left ventricle, which was hypo- or hyperechogenic, compared with the non-infarcted region.

Isolation of BMMNCs

Isolation of BMMNCs from the femoral and tibial bones of donor rats (n=10) was performed using a previously described method.[11] After an overdose of sodium pentobarbital (100 mg/kg, i.p.) the femora and tibiae were collected. Both ends of the bones were cut away from the diaphyses, the marrow cavities were flushed with Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) including 0.02M ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO, USA) and 5% fetal bovine serum (FBS; GIBCO, BRL Grand Island, NY, USA). The collected cells were transferred into a sterile tube containing 50 mL of culture medium (DMEM + 5% FBS + 10% EDTA). After two washes, the pellet was resuspended in 5 mL of culture medium. Mononuclear cells were isolated by density gradient centrifugation through Lympholyte-H (1.077 g/mL; Cedarlane, Hornby, Ontario, Canada) at 400 g for 30 min at room temperature and the interface cells were collected and washed twice in Ca^{2+}-Mg^{2+}- free Dulbecco's phosphate-buffered saline (PBS; GIBCO BRL Grand Island, NY, USA) containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA).

Labeling of BMMNCs

The cells were centrifuged at 400 g for 10 min using a medium without serum. The PKH26 dye (Sigma), a red fluorescent cell linker (4×10^{-6} M) was added and the cell suspension was incubated for 5 min at 25°C inverting the tube periodically. Then an equal volume of serum was added and the suspension was incubated for 1 min. After this incubation the sample was centrifuged twice at 400 g for 10 min at 25°C to remove cells from the staining solution and the pellet was resuspended in DMEM at a concentration of about 20×10^6 cells in 500 μL for transplantation. The stained sample was examined with flow cytometry to evaluate the fluorescence intensity and the percentage of marked cells. The purity of the labeled BMMNCs was evaluated by flow cytometric analysis before transplantation.

Seven days after transplantation, the percentage of labeled BMMNCs was evaluated in the BM of the rats by flow cytometric analysis.

Cell injections

Seven days after the injury, the recipient rats were prepared for the infusion of BMMNCs. The rats were anesthetized (ketamine, 2 g/Kg body weight i.p.) and the femoral vein surgically isolated and exposed. The BMMNC suspension (500 μL) containing a median of 20 (17-28) $\times10^6$ cells was infused using a tuberculin sterile syringe with a 23G needle. The wound was then closed with 5-0 vicryl sutures. The control rats were injected with normal saline (500 μL).

Pathology examination and histologic staining

Seven days after the BMMNC injection (2 weeks after the experimental injury), the rats were sacrificed with an overdose of sodium pentobarbital. The heart was exposed through a sternotomy,

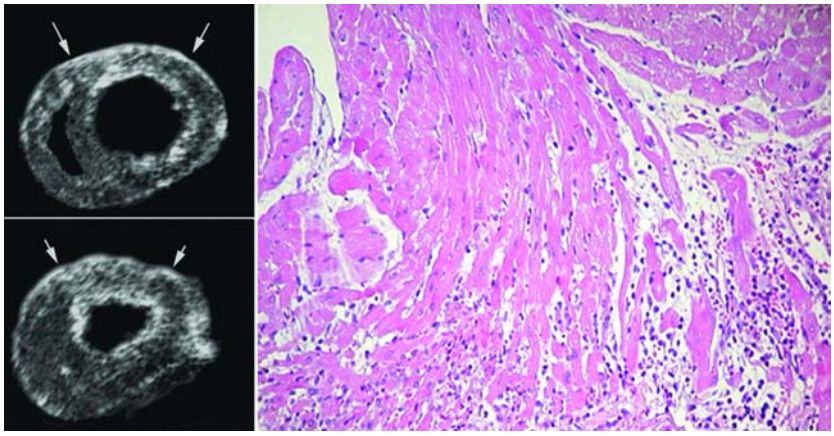

Figure 1. Left panel: 2D short axis echocardiographic images obtained in one of the studied animals at end diastole (upper panel) and at end systole (lower panel) showing the size of the injury (white arrows). Echocardiograms were performed in the acute setting (24 hours after the injury) with a 7.5 MHz mechanical probe; the focus depth and the sectorial angle were set at 3.0 cm and 60°, respectively. Right panel: Microscopic image (hematoxylin-eosin, 40×) showing freeze-thaw injuries. At the bottom and the lower-right corner of the figure the necrotic area with a dense lympho-granulocyte infiltrate can be identified. In the upper-left corner the left ventricular cavity is detectable.

arrested in diastole with an injection of potassium chloride (10 mEq) and the site of myocardial injury was identified. In each rat, transverse sections about 5 mm in thickness were made across the major axis of the liver, lung, kidney, thymus and spleen. Two transverse sections of infarcted myocardium and one of not-infarcted myocardium of about 3-5 mm in thickness were taken from the ventricular cavities. From each frozen block, 10 sections (5 μm in thickness) at 50 μm intervals were cut. Of every 10 sections, 1 was stained with hematoxylin and eosin, 4 were air-dried for 2 hours at room temperature in darkness and then mounted with a permanent aqueous mounting medium (Supermount, Biogenex, San Ramon, CA, USA) and 5 were stored for immunofluorescence and immunohistochemistry analysis.

To exclude the migration of labeled BMMNCs to their site of origin, the BM of all recipients was also harvested.

Estimates of the cells homing to tissues

Fluorescence was revealed with a reflected light fluorescence microscope (Olympus BX 60, Hamburg, Germany) at 1000X using a rhodamine filter (Olympus BP510-550). The homing of BMMNCs was estimated in each tissue by counting the number of red fluorescent cells in each section examined.

Identification of the injected population

Immunofluorescence and immunohistochemistry analyses were performed on the frozen sections of infarcted and non-infarcted myocardium in order to assess the Thy-1 FITC (clone OX-7; Becton Dickinson, San Jose, California, USA) and CD34 (clone ICO115; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) positive mononucleated cells, respectively. In order to visualize the heart tissue in the background when showing PKH26$^+$ cells, an indirect immunofluorescence stain was applied, using sarcomeric actin (clone α-Sr-1; DAKO, Glostrup, Denmark). The frozen sections were fixed with cool acetone (4°C) for 5 min, washed in PBS (pH 7.4, 0.1 M) and stained with specific antibodies. The antibodies were applied for 1 hour at a working dilution of 1:10 for Thy-1, 1:50 for CD34 and 1:20 for sarcomeric actin. Immunofluorescent-positive cells were identified using a reflected light fluorescence microscope (Olympus BX 60) at 1000X connected to a CCD camera (COHU 2200, Cohu Inc., San Diego, CA, USA). Images were post-processed by computer image analysis software (MacProbe v. 4.1, Perceptive Scientific Instruments Inc., League City, TX, USA). Diaminobenzidine (DAB) was used as the chromogen for CD34 immunostaining.

Statistics

Data were analyzed using computer statistical software (SPSS - Rel 6.1.1; SPSS Inc., Chicago, Illinois, USA). Infarcted areas are reported as mean ± standard deviation. The reproducibility of the experimental injury in all studied animals is reported as the coefficient of variation (CV) of the sizes of the infarcts obtained in the acute setting (24 hours after the freeze-thaw injury), as evaluated by echocardiography. Correlation between echographic and histologic estimates of infarcted areas was evaluated by linear regression analysis. A p value less than 0.05 is considered statistically significant.

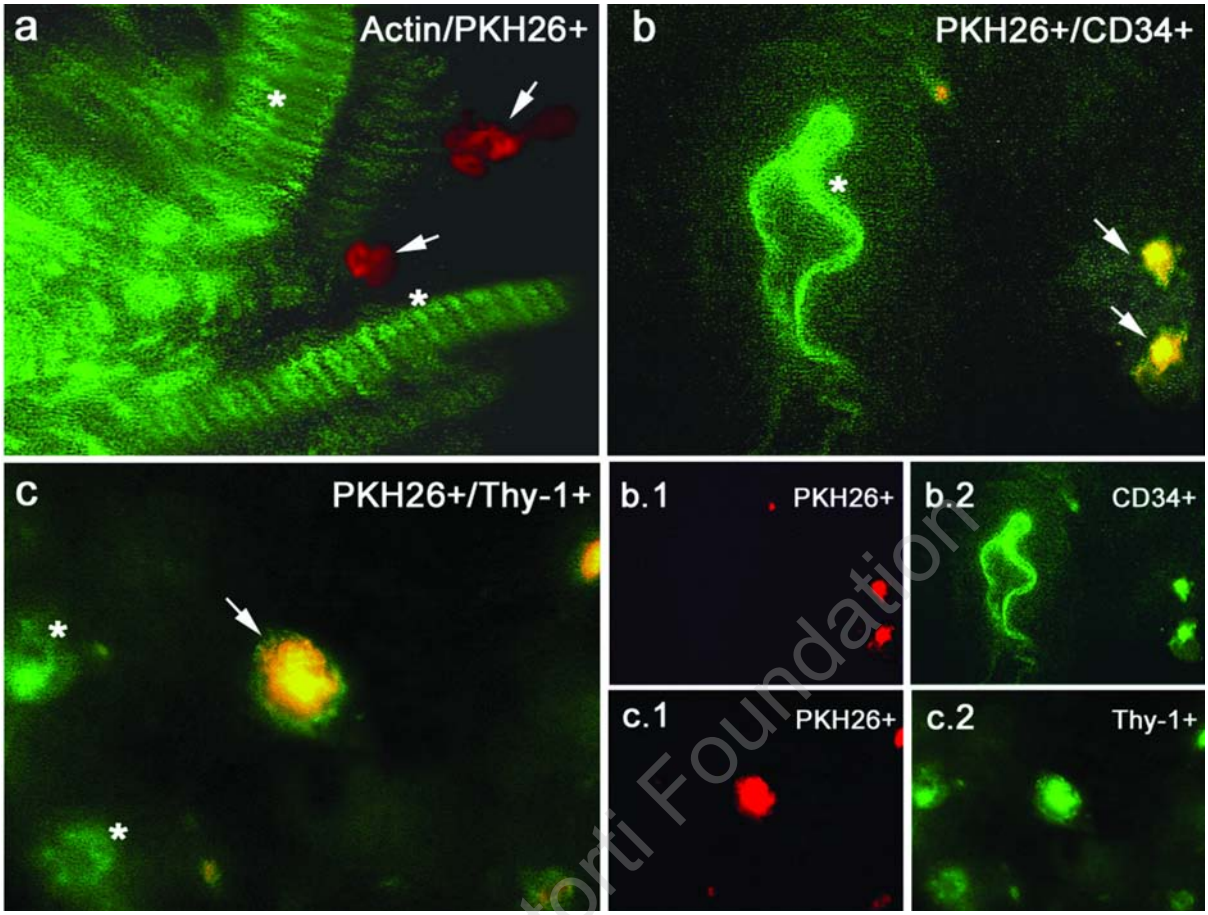

Figure 2. Panel a: double-fluorescence microscopy image showing sarcomeric actin(+), myocardiocytes (green fluorescence:*) and PKH26(+) cells (red fluorescence: arrows) within the site of the injury of the left ventricular wall (magnification 1000×). Panel b: double-fluorescence microscopy image showing PKH26 and CD34 co-expression (red-green fluorescence) in the injected BMMNCs (arrows), identified in the infarcted area. The asterisk indicates endothelial CD34+ cells used as an internal positive control (magnification 1000×). Panels b.1, b.2: single fluorescence images obtained from the same field as illustrated in panel b, showing PKH26+ (red fluorescence) and CD34+ (green fluorescence) cells (magnification 1000×). Panels c.1, c.2: single fluorescence images obtained from the same field as illustrated in panel c, showing PKH26+ (red fluorescence) and Thy-1+ (green fluorescence) cells (magnification 1000×).

Results

Echocardiography and histologic examination

On echocardiograms performed 24 hours after the injury, the left anterior myocardial wall of the rats showed akinetic and echodense areas that were considered as infarcted areas (Figure 1); the measured mean area of infarction in all studied animals was 21.2±3.4% of the transverse left ventricular free wall, with a CV of 0.16. Gross examination of the excised hearts at the end of the study (14 days after injury) showed clear myocardial scar formation; the histologic examination, conducted at the same level as the echocardiograms, confirmed the presence of a regular non-transmural scar of about 18.5±3.2% of the entire left ventricular free wall. Estimates of infarcted myocardium by echocardiography were in agreement with those obtained by histology ($r = 0.70$, $p = 0.02$). Ischemic infarcts usually have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, whereas freeze-thaw injuries consist of confluent necrosis in the subepicardium, with viable myocardium in the subendocardium (Figure 1). Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation and scarring after freeze-thaw injury are indistinguishable from ischemic myocardial infarction, making it a suitable model to study myocardial repair.[12-14]

Labeling and fate of injected BMMNCs

The median percentage of the PKH26-labeled BMMNCs before injection was 84% (range 70-91%) by flow cytometric analysis.

Table 1. Fate of transplanted bone marrow mononuclear cells (BMMNCs).

Target organ	*N. of sections examined Treated (n = 10)*	*% of sections with fluorescent BMMNCs*	*N. of sections examined Controls (n = 3)*	*% of sections with fluorescent BMMNCs*
Heart	120	45.83	36	0
Infarcted area	80	68.75	24	0
Non-infarcted area	40	0	12	0
Spleen	40	62.5	12	0
Liver	40	0	12	0
Kidney	40	0	12	0
Lung	40	0	12	0
Thymus	40	0	12	0
Total n. of sections	320	25	96	0

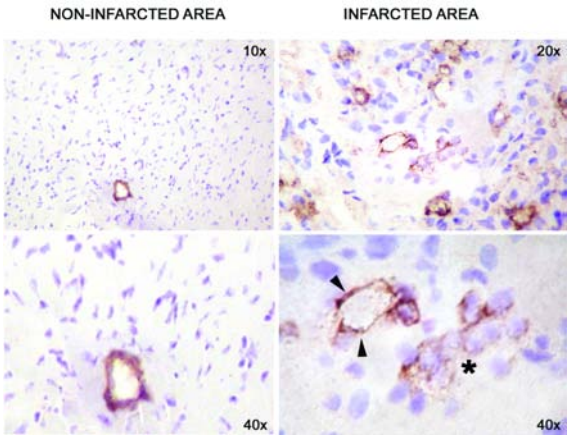

Figure 3. Microscopic images showing CD34 immunoreactivity. Non-infarcted area, left panels; infarcted area, right panels. Lower panels depict a higher magnification of a detail taken from the upper panels. CD34 brown immunoreactive mononuclear cells are evident only in the infarcted area. At 40x, some of them can be seen to be in contact with a vessel (arrows); the asterisk indicates a small cluster. No CD34$^+$ cells were found in the non-infarcted zone.

One week after the injection, BMMNCs red fluorescent cells were found only in the injured myocardium (Figure 2A), where 68.75% of the examined sections each contained a median of 8 (range 5-15) fluorescent cells and in the spleen, where a smaller number of fluorescent cells were found (median 3, range 1-3) in 62.5% of the examined sections. No red fluorescent cells were found in examined sections from any other target organs (Table 1). No fluorescence was found in negative controls. The median percentage of the labeled BMMNCs detected in the BM after transplantation was 0.8% (range 0.5-1.5%).

Identification of injected population

One week following the peripheral injection, PKH26$^+$ cells were found in infarcted areas (Figure 2A). Co-localization of PKH26 and CD34/Thy-1 demonstrated that hematopoietic progenitor cells had migrated to the injured heart via the peripheral circulation and localized just into infarcted areas (Figure 2B,C). The Thy-1$^+$ cells were homogeneously distributed in the infarcted areas with a decreasing epi-endocardial gradient superimposing the pattern of injury. The CD34$^+$ cells were identified close to the viable myocardium bordering the infarct zone (Figure 3). Positive cells were not identified outside the infarcted areas or in control rats.

Discussion

It is generally believed that the proliferative capacity of the adult mammalian myocardium is limited. Even though evidence exists for adult myocyte regeneration,[15] this concept remains highly controversial. Consequently, cardiomyocyte loss resulting from injury or disease is essentially irreversible. Cell therapy could, therefore, be a new approach to repair damaged tissue after myocardial infarction.

The cells to transplant and the source of the cells to isolate are important considerations.

During recent years, a number of *in vitro* and *in vivo* studies reported that BM contains pluripotent progenitor cells, capable not only of colonizing different tissues, but also of proliferating and transdifferentiating into cell lineages of host organs including osteocytes, chondrocytes, adipocytes, myocytes and even cardiomyocytes.[1,3,4,16-18]

Although the physiologic significance of such non-hematopoietic stem cells remains unknown, these pieces of evidence suggest that pluripotent stem cells may be used in adults as a way to recover lost functions of damaged tissues, with great advantages over embryonic stem cells. In fact, embryonic stem cells raise important ethical and safety issues regarding their teratogenic potential, whereas adult stem cells can be easily and safely used, particularly in an autologous setting.

Recently, it was demonstrated that intravenously injected bone marrow cells can home to damaged muscle of recipient mice and grow into new muscle fibers.[19-21] These results could have important consequences in view of the clinical use of BM cells in muscular dystrophy and in degenera-

tive cardiovascular diseases. Beltrami *et al.*[15] and Orlic *et al.*[22] showed that BM-derived stem cells, injected into the border of a myocardial infarct, homed to the infarcted zone and did not move into the remaining non-affected portion of the ventricular wall. A large number of stem cells seems to be required for the migration, multiplication and differentiation of these cells into the cell lineage of the damaged heart or other organs. In fact, the same authors demonstrated the presence of a subpopulation of myocytes that were not terminally differentiated in adult hearts. These myocytes re-entered the cell cycle and underwent nuclear mitotic division early after infarction. These data opened new therapeutic possibilities for patients affected by myocardial infarction (MI). In this regard, Strauer *et al.*[23] recently demonstrated that autologous BMMNCs can be safely administered through the coronary artery to patients 3 days after transmural MI. They treated 10 patients with standard pharmacologic treatment and additional cell therapy, obtaining a significant reduction in the extension of the infarct region (evaluated by ventriculography) and an improvement in cardiac function and contractility (evaluated by echocardiography) as compared to the corresponding results in the controls. This was the first clinical evidence of the safety and efficacy of BMMNCs in the treatment of MI and the results obtained by this group are very promising, although additional information about the cell subsets involved in myocardial repair must be collected in order to design more specific cell therapy protocols and to improve their efficacy.

One relevant issue is the origin of cycling myocytes in normal and diseased hearts. These proliferating cells could derive from resident cardiomyocytes or from circulating stem cells that reach the spared myocardium. However, in the absence of stimulation by several cytokines, the number of circulating stem cells is very low. The repair of the necrotic myocardium may involve interventions that promote the migration of endogenous, exogenous, or both types of stem cells to the infarcted region. Myocyte proliferation may be a component of the growth reserve of the human heart, involved in the mechanism through which the damaged myocardium could be repaired.

The presence of cell division in the non-diseased part of the heart suggests a continuous turnover of cells during the life span of the organism. Moreover, it can be argued that migration of myogenic precursors from extracardiac sites also occurs at low level in pathologic conditions such as myocardial infarction. This hypothesis is supported by recent studies demonstrating the presence of the Y chromosome in cardiomyocytes of human female allograft hearts transplanted into male patients.[24]

Furthermore, studies have demonstrated that mobilization of BM cells into the circulation and, presumably, their translocation to the infarcted portion of the heart can be obtained after granulocyte colony-stimulating factor (G-CSF) administration, leading to a significant magnitude of myocardial repair.[8]

Different administration routes have been proposed for cell delivery to the damaged myocardium, including intramyocardial[25] and intracoronary injection,[10] while few reports exist on intravenous injection of the cells.[26,27]

The approach of injecting cells through the circulation was used by Bittira and co-workers.[26] They focused their attention on the homing and short-term differentiation of mesenchymal stem cells (MSCs) in the heart, providing no information about migration to other target organs. Kawamoto and co-workers demonstrated that expanded endothelial progenitors derived *ex vivo* from human blood can be efficaciously injected intravenously into athymic nude rats. They also showed that the injected cells are capable of integrating and completing their differentiation in sites of neovascularization in the infarcted area.[27] In this study we demonstrated the feasibility of peripheral injection of a suspension of BMMNCs, containing adult stem cells from isogenic rat donors, into recipient rats with myocardial injury. Our results, which were obtained following a minimally invasive administration route, could have a positive impact on the development of therapeutic protocols.

To demonstrate the capability of the peripherally injected BMMNCs to migrate through the circulation to the site of damage, we set up a rat cryonecrosis model. This model has advantages and disadvantages compared with an alternative model based on the temporary ligation of the coronary artery. Although the latter more closely reproduces the pathologic events occurring immediately before and early after MI, in our opinion the cryonecrosis model has the advantage of providing a homogenous non-transmural scar in a defined location and of a reproducible size, which facilitates the association of the transplanted cells with the infarcted versus the non-infarcted areas.

To explain our data, we hypothesize that tissue injury leads to the priming of a cytokine cascade that acts as a chemoattractant for the infused cells. The specificity of this effect was confirmed by examining other possible target organs, including the lungs, liver, kidneys, spleen and thymus. No donor BMMNCs were found in any of these possible targets, except a small number in the spleen and the BM where, as expected from the hematopoietic origin of BMMNCs, some were found. In order to identify the cell population that migrates to the damaged myocardium, we stained the sections of infarcted and non-infarcted areas in both treated rats and controls with two markers for hematopoietic progenitor cells, Thy-1 and

CD34. The positivity for Thy-1 and CD34 antigens was observed just in the infarcted region, close to the viable myocardium. Therefore, we hypothesize that, 7 days after the injection, this population of cells had migrated to the scar tissue from uninjured myocardium bordering the infarcted zone. Despite these encouraging findings, much remains to be determined regarding the role played by chemoattractant factors and the interactions with the surrounding microenvironment. In fact, further studies, *in vivo* and *in vitro*, will be addressed to assessing which cytokines are responsible for the chemotaxis of the myogenic precursors and whether these cells are able to generate functional tissue capable of performing a cardiac-like duty cycle and adapting to the complex fiber geometry of the heart. Moreover, since the feasibility of our method of injection has been demonstrated, the goal of our further studies will be to identify the cells capable of regenerating damaged myocardium by comparing different BMMNC subsets.

In conclusion, our findings suggest that peripherally injected BMMNCs can traffic through the circulation to the site of tissue damage. This fact simplifies the strategy for cell implantation and supports the hypothesis that tissue damage, via the cytokine system, produces a chemotactic gradient that is sensed by circulating BMMNCs.

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Contributions

MMC: conception and design, development of the experimental model, analysis, echocardiograms and imaging and interpretation of data, drafting the article; LL: conception and design, isolation and labelling of cells, interpretation of data, drafting the article; RP: isolation and labeling of cells, drafting the article; AE: development of the experimental model, cell injections; SB: interpretation of data, critical revision of the article, final approval of the version; SF: conception and design, pathology examination, histology and immunofluorescence; UG: conception and design, pathology examination, histology and immunohistochemistry; RP: statistical analysis, drafting the article; GB: development of the experimental model, cell injections; AG: isolation and labeling of cells, drafting the manuscript; FM: interpretation of data, critical revision of the article, final approval of the version; PR: interpretation of data, critical revision of the article, final approval of the version

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received March 18, 2003; accepted April 11, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Studies on the use of hematopoietic stem cells for cardiac tissue repair have generally been based on direct intramyocardial injection.

What this study adds

In this animal model, a relatively high proportion of peripherally injected bone marrow cells were found to traffic through the circulation to the site of myocardial injury.