Cardiomyocyte Regeneration from Circulating Bone Marrow Cells in Mice

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ABSTRACT

We investigated the role of circulating bone marrow cells (BMC) in cardiomyocyte regeneration. BMC, isolated from transgenic mice expressing enhanced green fluorescent protein (GFP), were transplanted into lethally irradiated C57BL6 mice. Five weeks after bone marrow transplantation (BMT), flow cytometric analysis for GFP-positive cells confirmed reconstitution of transplanted bone marrow. Bone marrow transplant mice subsequently underwent left coronary artery ligation (myocardial infarction) or sham-operation, and were killed at 1 mo or 3 mo after operation. Infarct size was similar in bone marrow transplant mice at 1 mo ($47.1 \pm 5.9\%$) and at 3 mo ($45.3 \pm 7.8\%$), and echocardiography at 2 and 8 wk revealed decreasing left ventricular function. In infarcted heart, GFP-positive cells that expressed desmin and troponin T-C were identified by confocal microscopy. GFP and troponin T-C double-positive cells were predominantly in the peri-infarcted region (1 mo, 365 ± 45 cells/50 sections; 3 mo: 458 ± 100 cells/50 sections; $p < 0.05$ versus noninfarct, infarct, and sham-operated regions). Furthermore, BMC mobilization and differentiation into cardiomyocytes was found to be complete within 1 mo after myocardial infarction. These results demonstrate that circulating BMC undergo mobilization and differentiation in cardiac cells after myocardial infarction. Future studies are required to determine the molecular

signaling mechanisms responsible for this phenomenon. (*Pediatr Res* 54: 319–325, 2003)

Abbreviations

BM, bone marrow
BMC, bone marrow cells
BMT, bone marrow transplantation
BMT-MI mouse, bone marrow transplanted mouse with myocardial infarction
E-GFP, enhanced green fluorescent protein
G-CSF, granulocyte-colony stimulating factor
GFP, green fluorescent protein
IH, immunohistological
Lo, lost myocardium
LV, left ventricular
LVIDd, left ventricular internal dimension at end-diastole
LVIDs, left ventricular internal dimension at end-systole
LVEF, left ventricular ejection fraction
MI, myocardial infarction
MT, Masson's trichrome
PE, phycoerythrin
Re, remaining viable myocardium
SCF, stem cell factor
TXRD, Texas red

Clinical therapies for treatment of MI have undergone recent progress; however, the efficacy of these therapies is limited by rapid necrosis of the myocardium and postinfarct ventricular remodeling (1–3). The replacement of lost tissue with skeletal or cardiac myocytes, termed *cell therapy*, has been investigated as a potential means to regenerate infarcted myocardium and improve post-MI survival (4–7). Moreover, endothelial cells, originating in host BM, have been shown to participate in vasculogenesis (8–9). This mechanism of neovascularization after myocardial ischemia

or infarction has been extensively studied (10–13), and clinical trials are now ongoing to determine its efficacy in humans (14, 15). Recently, it has also been reported that BM contains pluripotent stem cells that are capable of differentiation into cardiomyocytes *in vitro* and *in vivo* (16–19). In our laboratory, we have established a murine whole BMT model in which murine marrow is reconstituted with BMC isolated from transgenic mice overexpressing E-GFP (20, 21). Using this model, we investigated the role of endogenous, circulating BMC in regeneration of cardiomyocytes after MI.

MATERIALS AND METHODS

All protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Insti-

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tutes of Health, and were approved by the Animal Experimental Ethical Review Committee of Nippon Medical School (Approval number 13–4). E-GFP transgenic mice were kindly donated by Dr. Okabe (Osaka University, Osaka, Japan).

Bone marrow cells harvest and transplantation. Two days before BMT, 8-wk-old donor E-GFP transgenic mice were administered fluorouracil (5-FU; Kyowa, Tokyo, Japan) at a dose of 150 mg/kg i.v. to remove cycling cells (22). BMC were isolated from the femur and tibia and placed into 0.1 M PBS. The cell suspension was passed through a 40- μ m filter (BD Biosciences, Franklin Lakes, NJ, U.S.A.), centrifuged, and resuspended in PBS at a concentration of 1×10^7 cells/mL.

Age-matched (8 wk) C57BL6 mice were irradiated with a lethal dose of 10 Gy. Mice then received 5×10^6 donor BM mononuclear cells *via* tail vein injection.

Flow cytometric analysis. Five weeks after BMT, reconstituted BM and peripheral blood (PB) cell surface markers were analyzed by flow cytometry (BD Biosciences). Erythrocytes were removed with lysing solution (Nichiyei, Tokyo, Japan), and BMC and PB cells were suspended in PBS and stained with PE-conjugated MAb. PE-conjugated MAb to Mac-1 (M1/70), Gr1 (RB6–8C5), CD45R/B220 (RA3–6B2), and Thy-1.2 (30-H12) were used to recognize macrophages, granulocytes, mature B lymphocytes, and T lymphocytes, respectively. Antibodies were purchased from BD PharMingen (San Diego, CA, U.S.A.). BMC or PB cells were incubated with each of these antibodies for 30 min on ice, and washed with PBS. After preparation, the percentages of GFP-positive cells and cells positive for both GFP and PE were analyzed.

Left coronary artery ligation model. Left coronary artery ligation was performed on 12 BMT mice and six age-matched C57BL6 mice, whereas six BMT mice underwent a sham-operation. Each mouse was anesthetized with intraperitoneal pentobarbital (50 mg/kg), intubated, and mechanically ventilated with room air using a Harvard ventilator (model 683). After a left thoracotomy, the pericardium was opened. Left coronary artery ligation or sham-operation was then performed, as per the procedure reported in detail by Michael *et al.* (23).

Analysis of myocardial function. At 2 and 8 wk after operation, BMT-MI mice and sham-operated mice underwent transthoracic echocardiography under anesthesia with intraperitoneal pentobarbital (50 mg/kg) using an Acuson SE-QUOIA 512 echocardiographic machine (Siemens Medical Solutions USA, Malvern, PA, U.S.A.). Two-dimensional short axis images were obtained at the level of mid-papillary muscle, and recorded. LVIDd and LVIDs were then measured, and LVEF was calculated as an index of contractile function.

Histologic examinations. One month after MI, at which time inflammatory cell infiltration into the site of MI and visceral pericardium disappears (24, 25), or 3 mo after MI, operated mice were killed with an overdose of intraperitoneal pentobarbital. Hearts were subsequently perfusion-fixed with 4% paraformaldehyde in 0.1 M PBS to remove circulating blood cells and to allow for proper fixation of myocardium for histology studies. Frozen tissue sections 2- μ m thick were cut on a cryostat, air-dried, and histologically examined. IH stain,

MT stain, and hematoxylin-eosin stain were performed in a series of transverse sections. The transverse axis of the mid-region was used to evaluate remaining viable (Re) and lost (Lo) myocardium in the left ventricular free wall (26–28), and the percentage infarct size was calculated as follows:

$$\% \text{ infarct size} = 100 \times \text{Lo}/(\text{Lo} + \text{Re})$$

Moreover, we divided the infarcted heart section into non-infarcted, infarcted, and peri-infarcted regions. Although the “at risk” area was uncertain, MT-stained slides allowed for clear recognition of the border between the infarct and noninfarcted area. The peri-infarcted region was defined as a scarred region within 100 μ m from the edge of the infarct border.

Immunohistological staining was also performed. A monoclonal desmin antibody (Novocastra, Newcastle, U.K.) (1:100 dilution), and a goat polyclonal troponin T-C antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) (1:250 dilution) were used for cardiac-specific markers. A monoclonal CD45.2 antibody (BD PharMingen) (1:100 dilution) and a rat macrophages/monocyte antibody (Cosmobio, Tokyo, Japan) (1:30 dilution) were used for white blood cell-specific markers. Mouse MAb were detected with a VECTOR M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA, U.S.A.). TXRD-conjugated donkey anti-goat IgG (Cortex Biochem, San Leandro, CA, U.S.A.) (1:250 dilution) and TXRD-conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR, U.S.A.) (1:150 dilution) were used for secondary antibodies.

Fluorescent analysis was undertaken with a confocal, laser scanning microscope (Leica TCSSP, Heidelberg, Germany). Cells with GFP and troponin T-C double-positive staining were defined as regenerated cardiomyocytes, whereas GFP-negative and troponin T-C-positive cells were defined as endogenous cardiomyocytes. Random fields (at least 600 fields in each area) were examined by a blinded operator for appropriate assessment.

Statistical analysis. All data are reported as mean $\pm$ SD. Results were analyzed by ANOVA (Scheffé’s posthoc analysis). Differences were considered statistically significant at $p < 0.05$.

RESULTS

BM reconstitution. The percentage of GFP-positive cells in recipient BM was 96.7% at 1 mo, 92.9% at 3 mo, and 90.8% at 6 mo after BMT, respectively. Moreover, GFP-positive cells within PB (96.2% at 1 mo after BMT) had differentiated into Mac-1/Gr1 ($23.4 \pm 0.9\%$), CD45R/B220 ($36.4 \pm 1.2\%$), and Thy-1.2 ($53.6 \pm 2.1\%$) lineages. The proportion of each lineage in recipient PB was similar to pre-BMT.

Left coronary artery ligation. Two BMT-MI mice died within 2 wk after operation. Six BMT-MI mice, three C57BL6/J mice with MI, and three sham-operated BMT mice were killed at 1 mo after operation. Four BMT-MI mice, three C57BL6 mice with MI, and three sham-operated BMT mice were also killed at 3 mo after operation. The mortality rate of this ligation model was lower than in the previous reports (23), though a thinned and scarred left ventricular free wall was confirmed under microscopic observation. Furthermore, the peri-infarcted region was clearly distinguishable (Fig. 1A).

Figure 1. Estimation of myocardial infarction. MT staining of an infarcted heart (A, original magnification: $\times 40$). Fibrous tissue is identified by blue staining. The peri-infarcted region is clearly identifiable. Echocardiographic records (B) and results (C) of BMT mice indicate that decreasing cardiac function is remarkable in MI group (arrowhead indicates akinesis of the left ventricular wall). * $p < 0.05$ vs S at the same phase; † $p < 0.05$ vs MI at 8 wk after operation. S, mice after sham-operation; MI, mice after myocardial infarction.

Infarct size (as percentage LV) in BMT-MI mice was $47.1 \pm 5.9\%$ at 1 mo, and $45.3 \pm 7.8\%$ at 3 mo (p value was not statistically significant).

Echocardiographic study. LVIDd, LVIDs, and LVEF were significantly different between BMT-MI mice and sham-operated mice. Furthermore, BMT-MI mice exhibited progressive impairments in echocardiographic parameters between at 2 and 8 wk after MI (Fig. 1, B and C).

GFP-positive cells accumulate in peripheral infarct. Although cardiomyocytes exhibited a low, nonspecific autofluorescence under excitation light, E-GFP allowed unambiguous identification of the location of BM-derived cells. Mobilized BMCs were detected in greatest concentration in the peri-infarcted region (Fig. 2, A–C) and within coronary vasculature (data not shown). GFP-positive cells, however, were rare in the other regions except as blood cells.

Figure 2. GFP-positive cells accumulate in peri-infarcted myocardium. Peri-infarcted region of C57BL6 mouse (A), peri-infarcted region of BMT mouse (B), and noninfarcted region of BMT mouse (C) are shown under excitation light. In noninfarcted myocardium, a GFP-positive cell that expresses CD45 is seen (original magnification: $\times 400$; scale bar: 50 μm). However, GFP and macrophage/monocyte (MAC/MONO) antigen (D) or CD45 (E) positive cells are scarcely observed in the peri-infarcted region (arrow: macrophage). Original magnification: $\times 400$; scale bar: CD45 50 μm , MAC/MONO 20 μm .

Figure 3. GFP-positive cells express desmin and troponin T-C. The peri-infarcted region at 1 mo is stained with desmin (A) and troponin T-C (B) antibodies (see “Materials and Methods”). In the overlay image, GFP-positive cells that express cardiac markers are yellow (original magnification: $\times 630$; scale bar: 20 μm).

To ensure that GFP-labeled cells in the heart were not inflammatory cells, we performed immunohistological staining with CD45 and macrophage/monocyte specific antibodies. As shown in Figure 2, D and E, inflammatory cells were scarce in number in the peri-infarcted region.

The mobilization and differentiation of BMC is completed within 1 mo after MI. Total cardiomyocyte number counted in

each section was $12,393 \pm 2,600$ cells (noninfarct region, $10,637 \pm 1,861$ cells; peri-infarct region, $1,519 \pm 303$ cells; infarct region 395 ± 245 cells) at 1 mo, and $11,519 \pm 1,735$ cells (noninfarct region, $9,084 \pm 1,556$ cells; peri-infarct region, $2,225 \pm 228$ cells; infarct region, 210 ± 44 cells) at 3 mo after MI. GFP-positive cells in the infarcted heart also co-expressed desmin and troponin T-C (Fig. 3). These cells were

Figure 4. Cardiomyocyte regeneration at 1 mo and at 3 mo after MI. Overlay views of noninfarct (*left*), peri-infarct (*mid*), and infarct (*right*) regions at 1 mo (*A*) and at 3 mo (*B*) are shown (GFP: green; troponin T-C: red; GFP and troponin T-C: yellow). (*C*) The number of regenerated cardiomyocytes in 50 sections is indicated. BMC mobilization and differentiation into cardiomyocytes are observed predominantly in the peri-infarcted region, and are almost completed within 1 mo after MI. *,† $p < 0.05$ vs Sham, Noninfarct, and Infarct at the same phase. *Noninfarct*, noninfarcted region; *Peri-infarct*, peri-infarcted region; *Infarct*, infarcted region; *Sham*, sham-operated region.

20–50 μm in length. The number of GFP and troponin T-C double-positive cells was highest in the peri-infarcted region (1 mo: 365 ± 45 cells/50 sections; 3 mo: 458 ± 100 cells/50 sections; $p < 0.05$ versus sham, noninfarct, infarct regions). However, the difference between the values in each region at 1 and 3 mo after MI was not statistically significant (Fig. 4). The percentage of newly formed cardiomyocytes for the entire LV was $0.04 \pm 0.02\%$ at 1 mo and $0.04 \pm 0.03\%$ at 3 mo after MI.

DISCUSSION

Recently, Jackson *et al.* (13) documented the regeneration of cardiac muscle from BM stem cells using a LacZ transgenic murine BMT model. However, animals were followed for only 1 mo, and quantitative data in each region was not shown. To our knowledge, this is the first study to document cardiomyocyte regeneration from endogenous BMC for more than 1 mo.

In our results, GFP and troponin T-C double-positive cells accumulated predominantly in the peri-infarcted region. Beltrami *et al.* (29) have reported that human cardiac myocytes also divide most in the region adjacent to the infarct after MI. These results suggest that repopulation of cardiomyocytes is targeted to repair the injured myocardium and its surroundings. Although some cytokines and/or growth factors, such as G-CSF and SCF, may be involved (26, 30), the mechanism by which communication occurs between BMC and cardiomyocytes has not yet been fully elucidated.

Cardiomyocyte regeneration from endogenous BMC is small in number ($0.04 \pm 0.02\%$ at 1 mo and $0.04 \pm 0.03\%$ at 3 mo), and is essentially complete within 1 mo after MI. Echocardiography revealed that newly replaced cardiomyocytes were not sufficient to improve cardiac function. Although this result can be expected, we believe that site-specific accumulation of circulating BMC and differentiation into cardiomyocytes are important events for certain clinical situations. Evidence that patients suffering angina before MI have better in-hospital prognosis and smaller infarcts has been previously reported (31–36). Orlic *et al.* (26) reported that G-CSF and SCF mediated mobilization of BM cells into infarcted hearts increased newly formed cardiomyocytes, and decreased mortality and infarct size. Although ischemic preconditioning and neovascularization likely play main roles in these clinical contexts, BMC-derived cardiomyocytes may also be important in the temporal repair of damaged cardiac tissues. Furthermore, it may be worth investigating whether there is an age-associated difference in cardiomyocyte regeneration.

Regarding potential problems and limitations of this experimental design, it is considerable that irradiation affected the recipient heart and/or other organs. Moreover, Lin *et al.* (37) have reported that circulating endothelial cells produced from transplanted marrow-derived cells display a greater proliferation rate than native cells. It is therefore possible that some differences exist between endogenous BMC and transplanted

BMC. These factors must be taken into consideration to accurately assess the results of this study.

CONCLUSION

In conclusion, cardiomyocyte regeneration by circulating BMC was observed largely in the peri-infarcted region, and was essentially complete by 1 mo post-MI. However, regenerated cardiomyocytes from BMC were small in number, and not sufficient to improve cardiac function. Further investigation is required to determine the molecular signals responsible for mobilization and differentiation of BMC after MI.

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