Implantation of Bone Marrow Mononuclear Cells Into Ischemic Myocardium Enhances Collateral Perfusion and Regional Function via Side Supply of Angioblasts, Angiogenic Ligands, and Cytokines

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Background—Bone marrow implantation (BMI) was shown to enhance angiogenesis in a rat ischemic heart model. This preclinical study using a swine model was designed to test the safety and therapeutic effectiveness of BMI.

Methods and Results—BM-derived mononuclear cells (BM-MNCs) were injected into a zone made ischemic by coronary artery ligation. Three weeks after BMI, regional blood flow and capillary densities were significantly higher (4.6- and 2.8-fold, respectively), and cardiac function was improved. Angiography revealed that there was a marked increase (5.7-fold) in number of visible collateral vessels. Implantation of porcine coronary microvascular endothelial cells (CMECs) did not cause any significant increase in capillary densities. Labeled BM-MNCs were incorporated into 31% of neocapillaries and corresponded to 8.7% of macrophages but did not actively survive as myoblasts or fibroblasts. There was no bone formation by osteoblasts or malignant ventricular arrhythmia. Time-dependent changes in plasma levels for cardiac enzymes (troponin I and creatine kinase-MB) did not differ between the BMI, CMEC, and medium-alone implantation groups. BM-MNCs contained 16% of endothelial-lineage cells and expressed basic fibroblast growth factor, vascular endothelial growth factor, angiopoietin 1 mRNAs, and their cardiac levels were significantly upregulated by BMI. Cardiac interleukin-1β and tumor necrosis factor-α mRNA expression were also induced by BMI but not by CMEC implantation. BM-MNCs were actively differentiated to endothelial cells in vitro and formed network structure with human umbilical vein endothelial cells.

Conclusions—BMI may constitute a novel safety strategy for achieving optimal therapeutic angiogenesis by the natural ability of the BM cells to secrete potent angiogenic ligands and cytokines as well as to be incorporated into foci of neovascularization. (Circulation. 2001;104:1046-1052.)

Key Words: angiogenesis ■ blood cells ■ transplantation ■ ischemia

Bone marrow (BM) stromal cells have many of the characteristics of stem cells for mesenchymal tissues. After BM transplantation, donor-derived cells have been found in multiple nonhematopoietic tissues, including vascular endothelial cells (ECs), skeletal muscle, liver, and bone. Endothelial progenitor cells (EPCs) were found in adult peripheral blood and mobilized from BM in response to tissue ischemia. Marrow stromal cells secrete a broad spectrum of angiogenic or antiangiogenic cytokines. Interleukin (IL)-1β was also reported to induce expression of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) in coronary microvascular endothelial cells (CMECs). Therapeutic availability of various angiogenic molecules has been reported in animal models or humans with ischemic heart disease. Recently, Kalka et al and Murohara et al reported that EPCs expanded from adult peripheral or cord blood improved capillary density in hindlimb ischemia. Autologous transplantation of BM cells was shown to enhance angiogenesis or improve cardiac function in a rat ischemic heart model. Because BM mononuclear cells (MNCs) contain various kinds of cell lineages, such as...
hematopoietic cells, fibroblasts, osteoblasts, and myogenic cells, as well as endothelial lineage, such mixed populations of BM-MNCs can work both beneficially and harmfully in ischemic myocardium. Therefore, this preclinical study using a swine model was designed to test therapeutic effectiveness and safety, including side effects.

Methods

Isolation of MNCs From BM
Miniswine (~20 kg) were anesthetized with ketamine hydrochloride followed by halothane. Premedication was not prescribed, and pigs were euthanized by removal of the myocardium. Cells (~25 mL) were aspirated from the ileum. MNCs were isolated by Percoll gradient (Lymphoprep, Nycomed) and labeled with green fluorescence cell linker (PKH2-GL, Sigma Chemical Co). This animal experiment was approved by the Animal Care Committee of Kansai Medical University.

BM Implantation Into Ischemic Myocardium
Immediately after aspiration of BM, the left anterior descending coronary artery (LAD) was ligated. Subsequent to ~60 minutes of observation for stabilization of arrhythmia, ligation was confirmed by angiography, and then RPMI medium (Gibco BRL) containing BM-MNCs (n=8) or CMECs (n=5) (total 106 cells, 25 sites<0.02 mL per site) or medium alone (control, n=8) was injected into the LAD risk area, including ischemic border and infarction portions, with a 26-gauge needle. Three weeks after BM implantation (BMI), regional blood flow was measured and angiography was performed. A fluid-filled catheter was introduced into the femoral artery, and dP/dt and left ventricular end-diastolic pressure (LVEDP) were analyzed.

CMEC Preparation
We used porcine CMECs as a mature EC control. We have established the preparation of CMECs from adult rat hearts and reported that CMECs express VEGF-2 and VEGF. CMECs cultured for 7 days were used for the experiment. The pigs used in this study originated from a closed colony (Geichingen strain, NIBS, Japan), and their MHC class was fixed on the homologous DRB1/DQA/DQB gene locus, suggesting that immunological rejection after transplantation is negligible.

Quantitative Angiographic Analysis
Numbers of visible branches of the left circumflex coronary artery (LCX) (>100 μm in diameter) and patent LAD were counted by use of 5-mm2 grids by at least 2 experienced cardiologists who were unaware of the group identity of the angiographic film. Interobserver variation was <5%.

Monitoring Cardiac Blood Flow
Echocardiographic studies were performed 60 minutes (baseline) and 3 weeks after LAD ligation with an Agilent Technology Sonos 5500 with an ultraband S4 sector transducer. The transducer was placed on a standoff positioned on the epicardium. Myocardial contrast echocardiography was performed with second harmonic technologies at a standoff positioned on the epicardium. Contrast agent (Levovist, Nihon Schering, 300 mg/10 seconds) was bolus-injected via the mid papillary muscle short-axis level. LV end-diastolic volume (LVEDV) normalized to body weight (LVEDV/BW; mL/kg) and ejection fraction (EF) were calculated by 2D echocardiography. Total LV area was manually traced at the short-axis level of the mid papillary muscle. The perfusion defect was expressed as a percentage of LV myocardial perfusion area.

Immunohistochemistry and Analyses of Vessel Numbers
Paraffin-embedded sections were treated with rabbit anti-factor VIII antibody (Dako). To detect the BM-derived cells labeled with green fluorescence, samples were snap-frozen and cut with a cryostat. These were incubated with anti-desmin (clone DE-R-11, Dako), anti-vimentin (clone V9, Dako), anti-macrophage (PM-2K), and anti–factor VIII antibody, followed by incubation with FITC- or TRITC-conjugated secondary antisera. The infarction area was evaluated by tetrazolium red staining. To analyze the vessels, 5 fields (5 mm2) were randomly chosen from infarct portions bordered by noninfarcted portions in the direction of the LCX. Researchers who were unaware of the group identity of the slides evaluated the density of arteries and capillaries in each field by counting vessels in 5 randomly chosen unit areas (500 μm2) by use of ocular micrometers (Olympus). The total number of vessels in 25 U areas (5 fields with 5 U areas in each field) were counted. Interobserver variation was <5%.

Immunocytchemistry
We performed immunocytochemical analyses using Dil-acetylated LDL (acLDL) incorporation and Ulex lectin binding as markers of EC lineage as described previously.

Quantification of mRNA Levels
The mRNA levels for VEGF (3.3 kb), basic fibroblast growth factor (bFGF) (2.8 kb), angiopoietin (Ang)-1 (4.8 kb), Ang-2 (2.8 and 2.3 kb), IL-1β (3.5 kb), and tumor necrosis factor (TNF)-α (2.5 kb) were evaluated by Northern blots using cRNA riboprobes. For Ang-2, a 2.8 kb of signal was analyzed. Human cDNA fragments encoding Ang-1 and Ang-2 were kindly provided by Dr Yancopoulos (Regeneron); bFGF, IL-1β, and TNF-α were obtained by reverse transcription–polymerase chain reaction. As an internal RNA control, U3 rRNA was examined with cDNA probe.

Analysis of Cardiac Enzymes
Blood was obtained from the jugular vein. Plasma levels of cardiac troponin I (cTnI) and creatine kinase (CK)-MB were assayed (Opus Plus Analyzer, Behring Diagnostics) as described.

Statistical Analysis
Statistical analyses were performed with a 1-way ANOVA followed by pairwise contrasts with Dunnett’s test. Data (mean±SEM) were considered statistically significant at a value of P<0.05.

Results

Incidence of Endothelial-Lineage Cells in BM-MNC and CMEC Characterization
Fluorescence-activated cell sorter (FACS) analysis (Figure 1A) indicated that 26±1.8% and 28±1.5% of BM-MNCs incorporated Dil-acLDL and bound Ulex lectin (n=5), respectively, and 16±1.2% of cells were positive for both markers. Endothelial-lineage cells were considered to be included in this fraction. CMECs exhibited a high ratio of Dil-acLDL incorporation (88%) or Ulex lectin binding (75%) and were therefore used as a control of mature ECs (Figure 1B). BM-MNCs were actively incorporated into network structures with human umbilical vein ECs (HUVECs), suggesting that BM-MNCs have the ability to form networks with cocultured mature ECs (Figure 1C).

Cardiac Function and Infarct Size
EF was improved significantly, by 48%, in the BM-MNC group, whereas in the CMEC- and medium-injection groups, EF did not change (Figure 2A). LV dP/dtmax and LVEDP deteriorated in all groups, but the extent (percent change) was significantly smaller in the BM-MNC group (Figure 2B and...
LVEDV/BW was increased in the CMEC- or medium-injection groups (28% to 33%, P<0.05), but not in the BM-MNC group (Figure 2D). There was no significant improvement in cardiac function between the CMEC- and the medium-alone injection groups.

Regional Myocardial Blood Flow and Infarction Area

The perfusion defect was not different from the baseline value 3 weeks after injection of CMEC or medium (arrows in Figure 3). In the BM-MNC group, the perfusion defect was markedly reduced by as much as 83% compared with baseline values. Infarct area assessed by tetrazolium red staining was decreased in the BM-MNC group (18.3±1.8%, P<0.001, n=7) compared with those in the CMEC- or medium-injection groups (23.6±2.0%, n=5, and 24.2±2.3%, n=7, respectively). Five animals were next examined for 12 weeks after BM-MNC injection. Each animal showed persistent improvement in cardiac function (EF, 45±2.2%, P<0.001) and perfusion (76±5.2%, P<0.001). During this long-term observation, all BMI-treated animals were alive and exhibited no malignant arrhythmia assessed by 24-hour Holter ECG once a week.

Coronary Angiography and Histological Analyses

Although the arrow in Figure 4 indicates the proximal end of the LAD just after ligation, the distal portion of the LAD was visible in all animals treated by BM-MNC, CMEC, or medium injection 3 weeks after ligation, suggesting that collateral capillaries that supply blood flow to the LAD were formed even in the control group. Numbers of visible collateral vessels (>100 μm in diameter) branching from the distal portion of the LCx in the direction of the infarct, however, were markedly increased in BM-MNC–treated animals compared with CMEC controls (5.4±0.3-fold, P<0.001). Tissue sections were stained for anti–factor VIII antibody (to detect endothelial cells). The numbers of capillaries were greater (2.1-fold, 50 μm, 2.7-fold 50 μm, P<0.001) in the BM-MNC group than in CMEC controls (Figure 5).

Incorporation of BM-Derived MNCs

We next examined whether BM-MNCs are incorporated into the capillary walls or differentiated to other linkage cells. As shown in Figure 6, desmin-positive myocytes were surrounded by vimentin-positive fibroblasts. Factor VIII–posi-

Figure 1. Flow cytometric analyses of BM-MNCs and CMECs and network formation with HUVECs. BM-MNCs (A) and CMECs (B) were analyzed by FACS using DiI-acLDL incorporation and Ulex lectin binding (n=5). BM-MNCs labeled with green fluorescence were cocultured with HUVECs at 1:1 cell number on Matrigel plates (C). Angiogenesis-like network was observed 12 hours after coculture.

Figure 2. Effects of BMI on cardiac function. BM-MNCs (n=8), CMECs (n=5), or medium alone (n=8) was injected into ischemic myocardium. EF, LVEDV/BW, dP/dt max, and LVEDP were calculated ∼60 minutes and 3 weeks after LAD ligation. % changes indicate differences between before and after values (60 minutes and 3 weeks). Results shown are mean±SEM.

*P<0.05, **P<0.01 vs before or control values.
tive cells indicated the presence of capillary ECs. PM-2K–positive macrophages were rare and mainly localized in fibrous regions. Green-labeled BM-MNCs corresponded primarily to factor VIII– and PM-2K–positive cells (28±3.6% and 8.7±1.8% of total positive cells) and were less differentiated into desmin- or vimentin-positive. We could not detect bone formation by osteoblasts or histologically atherosclerotic changes in coronary arteries, and there were no capillary vessels incorporating CMECs (data not shown).

BM-MNCs Supply Angiogenic Ligands and Cytokines

We examined the mRNA expression of bFGF, VEGF, Ang-1, and Ang-2. MNCs expressed mRNAs for bFGF>VEGF>Ang-1 in that order, but not Ang-2 (Figure 7B). Cardiac mRNA levels of bFGF and Ang-1 were upregulated for 14 days after BM-MNC injection, whereas VEGF was most abundant after 1 day and reverted to the baseline level after 7 days (Figure 7C). bFGF was detected in endothelial cells of capillary vessels as well as ischemic lesions (Figure 8A). The presence of bFGF (red label) corresponded to vessels incorporating BM-derived MNCs (green label).

We determined cardiac IL-1β and TNF-α mRNA levels 3 and 7 days after injection of BM-MNCs, CMECs, or medium (Figure 8B). Their levels were significantly increased 3 days after BM-MNC injection (8.7- and 5.6-fold, P<0.001 versus control) but not in CMEC- or medium-injection groups.

Figure 3. Effects of BMI on regional myocardial blood flow. BM-MNCs (n=8), CMECs (n=5), or medium alone (n=8) was injected into LAD risk area. Myocardial contrast echocardiography was performed ~60 minutes (baseline) and 3 weeks after LAD ligation. Areas of total LV and perfusion defect were manually traced in short-axis view at mid papillary muscle level. Perfusion defect (indicated by arrows) was expressed as percentage of total LV area.24 Results shown are mean±SEM. *P<0.01 vs prebaseline values.

Figure 4. Effects of BMI on angiographic collateral vessel formation. Angiographically visible branches extend from LCx and patent LAD. Arrow indicates proximal end of LAD 60 minutes after ligation.
Inflammatory Changes
Cardiac enzymes (cTnI and CK-MB) were measured to examine myocardial inflammation. cTnI was reported to be more sensitive than CK-MB. As shown in Figure 8C, cTnI and CK-MB levels were markedly increased 24 hours after LAD ligation, to a similar extent between the BM-MNC–, CMEC-, and medium-injection groups, and thereafter showed a similar time-dependent decrease. This suggested that infarctions of similar size were produced and that myocardial inflammation due to BMI is negligible or too little to be detected by plasma levels of cardiac enzymes.

Discussion
Regenerative stem cells are thought to be committed to differentiate exclusively into the tissues in which they reside, whereas recent reports have suggested that some ostensibly tissue-specific progenitors may have differentiation potential outside of their tissue of origin. After BM transplantation, donor-derived cells were found in multiple nonhematopoietic tissues. BM cells can differentiate into cardiomyocyte-like cells, and transplantation of BM cells was shown to enhance angiogenesis or improve cardiac function in a rat ischemic heart model. Because BM-MNCs contain various kinds of cell lineages, such as hematopoietic cells, fibroblasts, osteoblasts, and myogenic cells, as well as endothelial lineage, such mixed populations of BM-MNCs can work both beneficially and harmfully in ischemic myocardium. Therefore, this preclinical study in pigs was designed to test the therapeutic effectiveness and safety, including side effects.

The main findings of this study included that (1) autologous implantation of BM-MNCs induced an improvement in regional blood flow and cardiac function and decrease in infarction size; (2) BM-MNCs were incorporated into capillary vessel walls but did not actively survive as fibroblasts, myoblasts, or osteoblasts; (3) although fewer BM-MNCs survived as macrophages, myocardial inflammation was negligible when assessed by release of cardiac enzymes; and (4) BM-MNCs supplied angiogenic ligands (bFGF, VEGF, Ang-1) and cytokines (IL-1β and TNF-α). Thus, this preclinical study demonstrates that BMI effectively and safely induces neovascularization in ischemic myocardium by supplying angioblasts as well as angiogenic factors. Such angiogenic and antiapoptotic factors specific for endothelial cells (VEGF, Ang-1) released from BM-MNCs may contribute to the high survival ratio of endothelial-lineage cells. The observation that BM-MNC–derived fibroblasts are not detected in fibrous regions implies that the proliferation of fibroblasts originated predominantly from host myocardium. Taken together with the lack of bone formation by osteoblasts, we conclude that in ischemic myocardium, some surviving factors to stabilize BM fibroblasts or BM osteoblasts are lacking, and BM endothelial-linkage cells can effectively differentiate into mature cells.
CD34⁺, a marker of hematopoietic progenitor cells, is also found on endothelial cells in adults. Asahara et al. showed that MNC CD34⁺ cells from adult peripheral blood form endothelial colonies. Shi et al. reported that VEGF and bFGF caused differentiation of MNC CD34⁺ hematopoietic cells into ECs. In this study, we found that some neocapillaries actually incorporated BM-MNCs, and BM-MNCs synthesized bFGF, VEGF, or Ang-1. We also found that the cardiac expression of bFGF, VEGF, and Ang-1 were upregulated by BMI, and BM-MNCs functionally form a network with cocultured HUVECs. The observation that MNC CD34⁺ cells isolated from peripheral or cord blood more actively form cord-like structures by coculture with MNC CD34⁺ is also consistent with release of angiogenic ligands from MNC CD34⁺ cells. Thus, BMI using both CD34⁺ and CD34⁻ cells rather than implantation using only CD34⁺ cells would be more efficient for induction of neovascularization.

Marrow stromal cells secrete a broad spectrum of inflammatory angiogenic cytokines. We found that cardiac IL-1β and TNF-α levels were markedly increased by BM-MNC implantation but not CMEC or medium injection (Figure 8). Because IL-1β upregulates the expression of VEGF and VEGFR-2 in CMECs, and IL-1β and TNF-α are shown to have angiogenic activity, it is highly likely that such cytokines are involved in angiogenesis. Kobayashi et al. also reported the involvement of IL-1β in BM cell–induced angiogenesis in a rat ischemic heart. These findings suggest that implantation of BM-MNCs, but not mature endothelial cells, is a feasible source for therapeutic angiogenesis.

The present study demonstrated that BMI caused a marked increase in regional blood flow, although physiological collateral circulation is known to be poor in swine. Angiography indicated, however, that even in medium injection, the main LAD was visible, apart from the distal portion of the ligation end, via potential collateral vessels, suggesting that physiological collateral vessel formation after abrupt LAD closure is not so poor as to produce extensive irreversible damage. Considering that BMI caused a dramatically favorable effect on regional perfusion, it is conceivable that BM-MNCs stimulate collateral vessel formation much more rapidly than we had expected, leading to salvage of the expansion of risk area or a decrease in the infarct size. During the review process of our manuscript, it was shown that infusion of ex...
vivo expanded EPCs or MNC<sup>CD34+</sup> caused neovascularization<sup>33</sup> and prevention of myocyte apoptosis in ischemic myocardium<sup>34</sup> and that hematopoietic-lineage–negative BM cells differentiated into cardiomyocytes in ischemic myocardium.<sup>35</sup> These studies are consistent with our present results and suggest that BM into ischemic myocardium becomes a safe and feasible strategy for not only therapeutic angiogenesis but also therapeutic regeneration of cardiomyocytes.

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References

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