

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Profoundly Reduced Neovascularization Capacity of Bone Marrow Mononuclear Cells Derived From Patients With Chronic Ischemic Heart Disease

Christopher Heeschen, Ralf Lehmann, Jörg Honold, Birgit Assmus, Alexandra Aicher, Dirk H. Walter, Hans Martin, Andreas M. Zeiher and Stefanie Dimmeler

Circulation 2004;109;1615-1622; originally published online Mar 22, 2004;

DOI: 10.1161/01.CIR.0000124476.32871.E3

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2004 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circ.ahajournals.org/cgi/content/full/109/13/1615>

Subscriptions: Information about subscribing to *Circulation* is online at
<http://circ.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Profoundly Reduced Neovascularization Capacity of Bone Marrow Mononuclear Cells Derived From Patients With Chronic Ischemic Heart Disease

Christopher Heeschen, MD*; Ralf Lehmann, MD*; Jörg Honold, MD; Birgit Assmus, MD; Alexandra Aicher, MD; Dirk H. Walter, MD; Hans Martin, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Cell therapy with bone marrow–derived stem/progenitor cells is a novel option for improving neovascularization and cardiac function in ischemic heart disease. Circulating endothelial progenitor cells in patients with coronary heart disease are impaired with respect to number and functional activity. However, whether this impairment also extends to bone marrow–derived mononuclear cells (BM-MNCs) in patients with chronic ischemic cardiomyopathy (ICMP) is unclear.

Methods and Results—BM-MNCs were isolated from bone marrow aspirates in 18 patients with ICMP (ejection fraction, $38\pm 11\%$) and 8 healthy control subjects (controls). The number of hematopoietic stem/progenitor cells ($CD34^+/CD133^+$), $CD49d^+$ (VLA-4) cells, and $CXCR4^+$ cells did not differ between the 2 groups. However, the colony-forming capacity of BM-MNCs from patients with ICMP was significantly lower compared with BM-MNCs from healthy controls (37.3 ± 25.0 versus 113.8 ± 70.4 granulocyte-macrophage colony-forming units; $P=0.009$). Likewise, the migratory response to stromal cell–derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF) was significantly reduced in BM-MNCs derived from patients with ICMP compared with BM-MNCs from healthy controls (SDF-1, 46.3 ± 26.2 versus 108.6 ± 40.4 cells/microscopic field, $P<0.001$; VEGF, 34 ± 24.2 versus 54.8 ± 29.3 cells/microscopic field, $P=0.027$). To assess the in vivo relevance of these findings, we tested the functional activity of BM-MNCs to improve neovascularization in a hindlimb animal model using nude mice. Two weeks after ligation of the femoral artery and intravenous injection of 5×10^5 BM-MNCs, laser Doppler–derived relative limb blood flow in mice treated with BM-MNCs from patients with ICMP was significantly lower compared with mice treated with BM-MNCs from healthy controls (0.45 ± 0.14 versus 0.68 ± 0.15 ; $P<0.001$). The in vivo neovascularization capacity of BM-MNCs closely correlated with the in vitro assessment of SDF-1–induced migration ($r=0.78$; $P<0.001$) and colony-forming capacity ($r=0.74$; $P<0.001$).

Conclusions—BM-MNCs isolated from patients with ICMP have a significantly reduced migratory and colony-forming activity in vitro and a reduced neovascularization capacity in vivo despite similar content of hematopoietic stem cells. This functional impairment of BM-MNCs from patients with ICMP may limit their therapeutic potential for clinical cell therapy. (*Circulation*. 2004;109:1615-1622.)

Key Words: stem cells ■ progenitor cells ■ angiogenesis ■ vasculogenesis ■ stromal cell–derived factor-1

Despite improved pharmacological therapy, congestive heart failure remains the leading cause of cardiovascular mortality in the United States and Europe.¹ Cell therapy is a promising novel option to improve vascularization or cardiac regeneration.² In animal models, bone marrow–derived stem/progenitor cell infusion improves cardiac function and neovascularization after myocardial infarction.^{3–6} The bone marrow contains different types of stem cells. Hematopoietic stem/progenitor cells, defined as $CD34^+$ cells in humans or

$c-kit^+/sca-1^+$ cells in mice, are incorporated into newly formed blood vessels, augment neovascularization, and were shown to give rise to cardiomyocytes.^{3,5} Moreover, human bone marrow–derived mesenchymal cells or isolated side population cells engraft and differentiate to a cardiomyocyte phenotype in murine hearts.^{7,8} Finally, adult circulating endothelial progenitor cells can give rise to new blood vessels and differentiate to cardiac myocytes in vitro.^{9,10} Recent clinical studies provide further evidence for a promising

Received July 21, 2003; de novo received October 13, 2003; revision received December 29, 2003; accepted January 5, 2004.

From Molecular Cardiology, Department of Internal Medicine IV, and Hematology, Department of Internal Medicine III (H.M.), University of Frankfurt, Frankfurt, Germany.

*The first 2 authors contributed equally to this work.

Correspondence to Stefanie Dimmeler, PhD, Molecular Cardiology, Department of Internal Medicine IV, University of Frankfurt, Theodor Stern-Kai 7, 60590 Frankfurt, Germany. E-mail dimmeler@em.uni-frankfurt.de

© 2004 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000124476.32871.E3

improvement of cardiac function after intracoronary infusion of bone marrow–derived cells in patients with acute myocardial infarction.^{11–13}

Previous studies demonstrate that risk factors for coronary artery disease correlate with a reduced number and functional activity of circulating endothelial progenitor cells.¹⁴ Moreover, diabetic patients showed impaired proangiogenic and colony-forming activity of circulating endothelial progenitor cells.^{15,16} The reduction of circulating progenitor cells in the peripheral blood may have different reasons, including induction of cell death, enhanced homing, or inhibition of differentiation. Alternatively, the reduced number and functional activity could be a result of an impaired mobilization of progenitor cells from the bone marrow. Because the regulation of stem/progenitor cells in the bone marrow of patients with heart disease has not yet been elucidated, we determined the number and the functional activity of stem/progenitor cells from bone marrow aspirates isolated from patients with chronic ischemic cardiomyopathy (ICMP).

Methods

Study Population

We collected bone marrow aspirates from a total of 8 healthy control subjects (controls) and 18 patients with stable coronary heart disease and a history of myocardial infarction at between 18 and 75 years of age. Patients had to have regional wall motion abnormalities, a patent native vessel, coronary artery bypass graft, or collateral artery. Because myocardial ischemia is known to mobilize bone marrow–derived progenitor cells, patients with evidence for inducible or resting myocardial ischemia during the previous 4 weeks were excluded. Further exclusion criteria were the presence of active or chronic infection, operations or trauma within the previous 2 months, evidence for malignant diseases, active gastrointestinal bleeding, uncontrollable hypertension >160/100 mm Hg, stroke within the previous 2 years, arteriovenous aneurysm, renal or hepatic insufficiency, thrombocytopenia with platelet counts <100 000/ μ L, anemia with hemoglobin <8.5 g/dL, mental retardation, enrollment in another clinical trial, or unwillingness to participate. We also excluded pregnant and premenopausal women. The ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Bone Marrow Mononuclear Cells

A total of 50 mL of bone marrow aspirate was obtained from each participant. Bone marrow–derived mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation. After 2 washing steps, cells were resuspended in 10 mL X-vivo 10 medium (Cambrex). The cell suspension consists of heterogeneous cell populations, including hematopoietic progenitor cells.

Flow Cytometry Analysis of BM-MNCs

For the identification of hematopoietic and mesenchymal stem/progenitor cell populations, we used directly conjugated antibodies against human CD45 (Becton Dickinson), CD34 (FITC-labeled; BD Pharmingen), CD133 (APC-labeled; BD Pharmingen), CD14 (FITC-labeled; BD Pharmingen), CXCR4 (APC-labeled, BD Pharmingen), and CD49d (APC-labeled; BD Pharmingen). The lineage panel was obtained from BD Pharmingen (containing FITC-labeled CD3, CD14, CD16, CD19, CD20, and CD56) and was completed by also using directly FITC-conjugated antibodies against CD15 and glycoprotein A (both from BD Pharmingen).

Colony-Forming Unit Assay

BM-MNCs (1×10^5 per dish) were seeded in methylcellulose plates (Methocult GF H4535 including stem cell factor, granulocyte colony–stimulating factor, granulocyte-macrophage colony–stimulating factor, interleukin-3, and interleukin-6; CellSystems). Plates were studied under phase-contrast microscopy, and granulocyte-macrophage colony–forming units (CFU-GM; colonies >50 cells) were counted after 14 days of incubation by 2 independent investigators.

Assessment of Migratory Capacity of BM-MNCs

A total of 1×10^6 BM-MNCs were resuspended in 250 μ L X-Vivo and placed in the upper chamber of a modified Boyden chamber filled with Matrigel (BioCoat invasion assay, 8- μ m pore size, Becton Dickinson Labware). Then, the chamber was placed in a 24-well culture dish containing 500 μ L endothelial basal medium supplemented with 10% fetal calf serum and singlequots. For some experiments, 50 ng/mL vascular endothelial growth factor (VEGF) or 100 ng/mL stromal cell–derived factor 1 (SDF-1) was added. After 24 hours of incubation at 37°C, transmigrated cells were counted by 2 independent investigators.

Hindlimb Ischemia Model

The neovascularization capacity of BM-MNCs was investigated in a murine model of hindlimb ischemia by use of 8- to 10-week-old athymic NMRI nude mice (Jackson Laboratory, Bar Harbor, Me) weighing 18 to 22 g. The proximal portion of the femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were ligated with 7-0 silk suture. All arterial branches between the ligation were obliterated with an electrical coagulator. The overlying skin was closed with 3 surgical staples. After 24 hours, BM-MNCs were injected intravenously.

Limb Perfusion Measurements

After 2 weeks, we measured ischemic (right)/normal (left) limb blood flow ratio with a laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System, moorLDI-Mark 2, Moor Instruments). Before scanning was initiated, mice were placed on a heating pad at 37°C to minimize variations in temperature. After two recordings of laser Doppler color images, the average perfusions of the ischemic and nonischemic limb were calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

Histological Evaluation

Tissue vascularization was determined in 5- μ m frozen sections of the adductor and semimembranous muscles from the ischemic and the nonischemic limbs. Endothelial cells were stained with FITC-labeled monoclonal antibody directed against CD146 (Chemicon). Capillary density is expressed as number of capillaries per myocyte. Human BM-MNCs were identified by costaining with HLA-A,B,C-APC-labeled antibodies (BD Pharmingen) and CD146-FITC-labeled antibodies.

Statistical Analysis

If not stated otherwise, results for continuous variables are expressed as mean \pm SD. Comparisons between groups were analyzed by *t* test (2-sided) or ANOVA for experiments with >2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with the *t* test (2-sided) with Bonferroni adjustment. Comparison of categorical variables was generated by the Pearson χ^2 test. After blind assessment of the in vitro characteristics of the BM-MNCs, test results were merged with the results from the in vivo studies. To identify in vitro determinants of BM-MNCs for their in vivo neovascularization capacity, a multivariate linear regression analysis was performed. Probability values <0.05 were considered statistically significant. All analyses were performed with SPSS 11.5 software.

TABLE 1. Baseline Characteristics

| | Patients With ICMP (n=18) | Healthy Controls (n=8) | <i>P</i> |
|--|------------------------------|---------------------------|----------|
| Age, y | 59.4±13.4 | 32.1±4.6 | <0.001 |
| Male sex, % | 88.9 | 87.5 | 0.65 |
| Hypertension, % | 57 | 0 | <0.001 |
| Hypercholesterolemia, % | 70 | 25 | 0.03 |
| Diabetes, % | 17 | 0 | <0.001 |
| Smoking, % | 82.4 | 37.5 | <0.001 |
| Family history for CHD, % | 52.9 | 25.0 | 0.21 |
| CAD (1/2/3-vessel disease), % | 17.6/52.9/29.5 | NA | ... |
| History of CAD, % | 100 | NA | ... |
| Infarct territory (anterior/inferior), % | 65/35 | NA | ... |
| Ejection fraction, % | 37.8±11.1 | 62.6±3.1 | <0.001 |
| Medication on discharge, % | | | |
| Aspirin | 100 | 0 | ... |
| Clopidogrel | 100 | 0 | ... |
| ACE inhibitor | 100 | 0 | ... |
| β-Blocker | 100 | 0 | ... |
| Statin | 100 | 0 | ... |
| C-reactive protein, mg/L | 3.0±2.5 | 2.9±1.7 | 0.51 |
| White blood cell count, ×10 ⁹ /μL | 7.9±3.7 | 6.8±2.9 | 0.85 |

CHD indicates coronary heart disease; CAD, coronary artery disease; and NA, not applicable.

Results

The characteristics and the functional activity of BM-MNCs were investigated in bone marrow aspirates from 18 patients with ICMP and 8 healthy controls. The baseline characteristics of the study population are depicted in Table 1.

Number and Migratory Potential of Bone Marrow Cells

To investigate whether the number of stem/progenitor cells is reduced in BM-MNCs of patients with ICMP relative to healthy controls, we determined the number of hematopoietic stem/progenitor cells characterized by the expression of the marker protein CD34. The number of CD34⁺/CD45⁺ BM-MNCs was similar in both healthy controls and patients with ICMP (n=8 healthy controls; n=18 patients with ICMP) (Figure 1a). Moreover, the more immature subset of hematopoietic progenitor cells defined as CD133⁺/CD34⁺ cells and uncommitted lineage⁻/CD34⁺ cells, respectively, did not differ between BM-MNCs from patients with ICMP and BM-MNCs from healthy controls. Likewise, mesenchymal stem cells, which were defined by the absence of staining for CD14, CD34, and CD45, were similar in the 2 groups. Mesenchymal stem cells represented 1.90±4.75% of BM-MNCs for patients with ICMP, compared with 1.08±1.36% of BM-MNCs for healthy controls (*P*=0.51).

The functional activity of stem/progenitor cells in bone marrow aspirates was further determined by measuring the colony-forming activity and migration. Interestingly, BM-MNCs from patients with ICMP showed a significantly reduced number of colony-forming units compared with

BM-MNCs from healthy controls (37.3±25.0 CFU-GM per dish versus 113.8±70.4 CFU-GM per dish; *P*=0.009) (Figure 1b). The migratory response to SDF-1 and VEGF was also significantly reduced in BM-MNCs derived from patients with ICMP compared with BM-MNCs from healthy controls (VEGF, 34 000±24 200 versus 54 800±29 300 migrated cells, *P*=0.027; SDF-1, 46 300±26 200 versus 108 600±40 400 migrated cells, *P*<0.001) (Figure 1c). The colony-forming capacity closely correlated with the migratory response to SDF-1 (*r*=0.65; *P*=0.001) but not with the migratory response to VEGF (*r*=0.21; *P*=0.34).

CXCR4 is the receptor for SDF-1, which also plays a crucial role for basal and VEGF-induced cell migration. To analyze whether a dysregulated expression of the CXCR4 receptor accounts for the impaired migratory response, we determined CXCR4 surface expression by flow cytometry. The proportion of CXCR4⁺ lymphocytes (healthy controls, 16.66±3.96% of lymphocytes versus ICMP, 14.33±4.66%; *P*=0.57) and CXCR4⁺/CD34⁺ lymphocytes (healthy controls, 0.26±0.19% versus ICMP, 0.33±0.45%; *P*=0.62) was similar for both groups. Furthermore, we determined the extent of CXCR4 expression as the geometric mean of the logarithmic fluorescence (mean log fluor). The expression of CXCR4 did not differ between BM-MNCs from healthy controls and BM-MNCs from patients with ICMP (healthy, 21.47±14.64 arbitrary units for mean log fluor versus ICMP, 30.12±23.52), even when subpopulations (eg, CD34⁺/CXCR4⁺) were analyzed (data not shown). Finally, expression of the integrin CD49d was not different in BM-MNCs from patients with ICMP compared with BM-MNCs from healthy controls (healthy controls, 6.66±4.72 versus ICMP, 5.29±3.11).

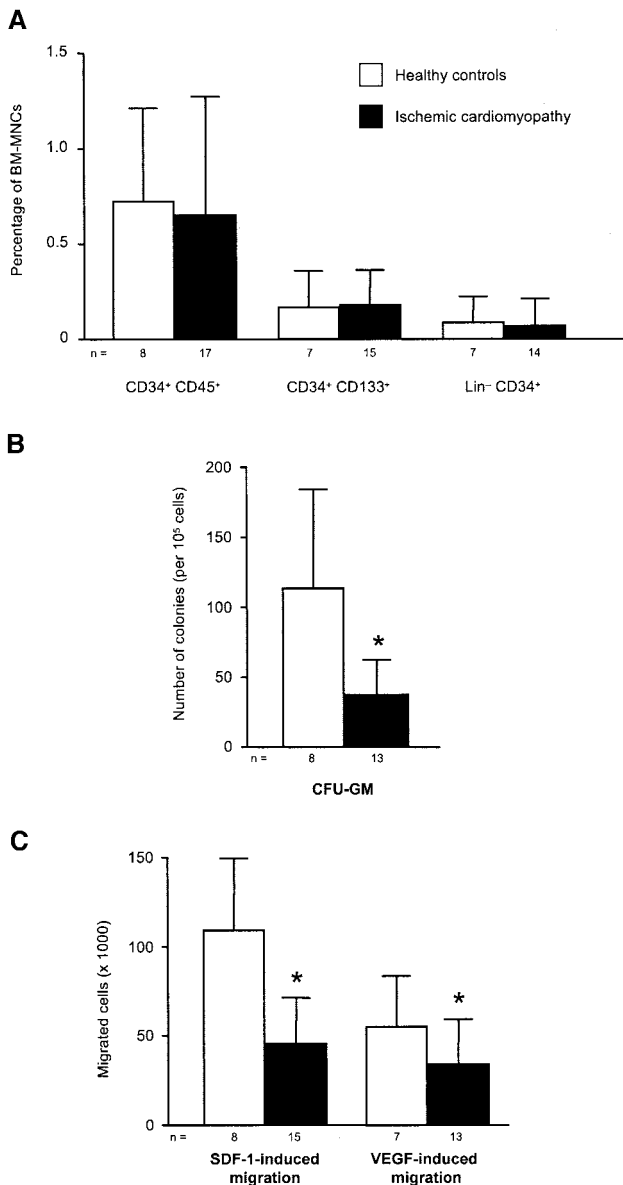


Figure 1. A, Flow cytometry analysis for stem/progenitor markers in BM-MNCs from healthy controls and patients with stable ischemic cardiomyopathy. No significant differences were observed between the 2 groups. B, Functional activity of BM-MNCs from healthy controls and patients with stable ischemic cardiomyopathy. Number of colony-forming units per 5×10^5 BM-MNCs was determined after 14 days. Migratory capacity of BM-MNCs in response to SDF-1 and VEGF was assessed using BioCoat invasion assay. * $P < 0.01$ vs healthy controls.

Effect of Bone Marrow Cells on Neovascularization After Hindlimb Ischemia

After induction of hindlimb ischemia in nude mice, BM-MNCs from healthy controls were injected intravenously (5×10^4 , 5×10^5 , or 5×10^6 cells/mouse; $n = 5$ per group). After 2 weeks, the effect of cell therapy on neovascularization was assessed by laser Doppler imaging. In accordance with previous studies, BM-MNCs from healthy controls significantly improved neovascularization (Figure 2a). The maximal therapeutic effect with BM-MNCs derived from healthy controls was achieved at a concentration of 5×10^5 cells/

mouse (data not shown). Therefore, this dose was used for comparison with BM-MNCs derived from patients with ICMP.

Animals treated with 5×10^5 BM-MNCs from patients with ICMP showed a significantly reduced improvement of neovascularization compared with 5×10^5 BM-MNCs from healthy controls (0.45 ± 0.14 versus 0.68 ± 0.15 ; $P < 0.001$; $n \geq 8$ per group) (Figure 2a). Consistently, histological assessment of the skeletal muscles revealed that BM-MNCs from healthy controls more efficiently stimulated tissue neovascularization. Capillary density was significantly higher in mice treated with BM-MNCs from healthy controls compared with mice treated with BM-MNCs from patients with ICMP ($185 \pm 88\%$ versus $116 \pm 31\%$ of control, $P < 0.01$; $n \geq 6$ per group). Moreover, we determined the incorporation of BM-MNCs into the capillaries by immunostaining of the tissue sections using antibodies directed against human HLA and the panendothelial marker CD146. The number of human cells, which coexpress endothelial marker proteins, was significantly reduced when BM-MNCs from patients with ICMP were infused compared with BM-MNCs from healthy controls (Figure 2b). To also assess the homing of human BM-MNCs into the ischemic tissue, we analyzed sections at an earlier time point (7 days after ischemia). Again, the number of human HLA-positive cells was significantly lower when BM-MNCs from patients with ICMP were infused (2.7 ± 2.3 versus 7.5 ± 4.0 cells per high-power field; $n = 9$ sections per group; $P < 0.01$). These data suggest that BM-MNCs from patients with ICMP suffer from both a marked impairment in their capacity to home to sites of ischemia and in their ability to incorporate and differentiate into proliferating vascular structures.

Independent Predictors for the Functional Activity of BM-MNCs

SDF-1-induced migration but not basal migration ($r = 0.38$; $P = 0.061$) or VEGF-induced migration ($r = 0.35$; $P = 0.078$) correlated with the functional improvement of neovascularization in the hindlimb ischemia model ($r = 0.78$; $P < 0.001$) (Figure 3a). The colony-forming activity of the BM-MNCs was also significantly associated with the neovascularization capacity of the cells ($r = 0.74$; $P < 0.001$) (Figure 3b). Several clinical baseline characteristics of the patients were also associated with the therapeutic effect of the BM-MNCs in the murine model of hindlimb ischemia (Table 2, left column), but all variables except for SDF-1-induced migration lost their level of statistical significance if the analysis was restricted to patients with ICMP (Table 2, middle column).

To identify independent predictors for the functional activity of BM-MNCs, we performed a multivariate linear regression analysis including baseline characteristics (with $P = 0.10$ necessary to enter a variable into the model). As depicted in Table 2 (right column), the migratory capacity of the BM-MNCs remained the only independent predictor of the neovascularization capacity as assessed in the model of hindlimb ischemia. The only other independent predictor was the colony-forming capacity, although the association fell short of achieving statistical significance.

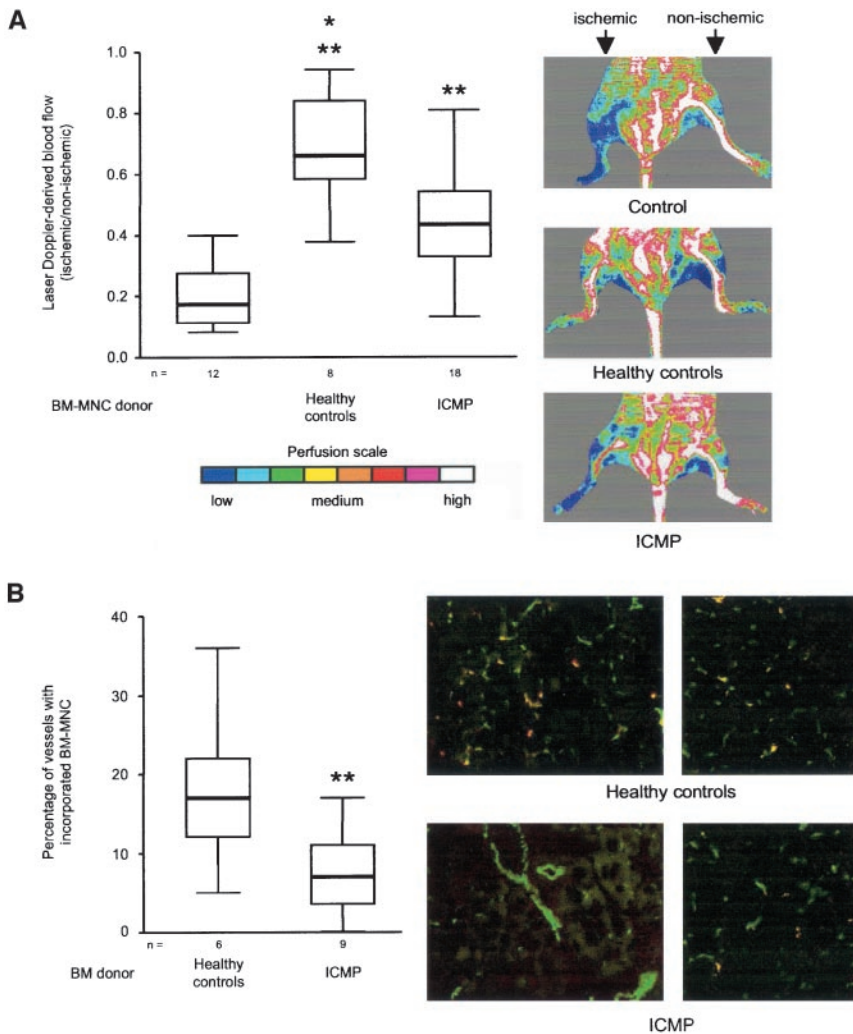


Figure 2. Improvement of postnatal neovascularization by intravenous injection of BM-MNCs in a murine model of hindlimb ischemia. a, Laser Doppler-derived relative blood flow in mice 2 weeks after induction of hindlimb ischemia. Untreated animals served as a negative control. Other animals received 5×10^4 BM-MNCs derived from healthy controls and patients with stable ischemic cardiomyopathy, respectively. Representative laser Doppler scans for 3 groups are depicted. Arrows indicate ischemic and nonischemic limb. Perfusion signal is subdivided into 8 different intervals, each displayed as a separate color. Low or no perfusion is displayed as dark blue; highest perfusion interval is displayed as white. b, Incorporation of human BM-MNCs into vascular structures of ischemic hindlimbs was assessed on day 14 after ligation of femoral arteries. Incorporated human BM-MNCs were identified by costaining for panendothelial marker CD146 and anti-human HLA. * $P < 0.01$ vs untreated; ** $P < 0.01$ vs healthy controls.

Discussion

The data of the present study demonstrate that mononuclear cells isolated from the bone marrow of patients with ischemic cardiomyopathy have a significantly reduced activity for promoting neovascularization in nude mice after induction of hindlimb ischemia. BM-MNCs have been used in first clinical trials to improve cardiac regeneration in patients with acute myocardial infarction^{12,13} and in patients with chronic ischemic heart disease.^{19,20} The most potent subpopulation(s) within the bone marrow, which is capable of improving neovascularization and/or cardiac regeneration, has not been clearly defined to date. Several experimental studies suggest that most likely, multiple distinct cell populations of the BM-MNC fraction are capable of contributing to vessel formation and differentiating into cardiac myocytes. Importantly, in the present study, we did not observe any differences in the number of hematopoietic stem/progenitor cells in the bone marrow. In the absence of specific markers for mesenchymal stem cells, we used a negative selection for the identification of mesenchymal stem cells as previously described.²¹ However, the number of mesenchymal stem cells, defined by negative staining for CD14, CD45, and CD34, was also similar in patients with ICMP and healthy controls. On the basis of these data, it is tempting to speculate that the

reduced number of circulating progenitor cells in patients with ischemic heart disease¹⁴ is not related to a reduced number of stem/progenitor cells in the bone marrow rather than to an impaired mobilization from the bone marrow. Indeed, lack of endothelium-derived nitric oxide, which is characteristic for patients with coronary artery disease, resulted in a defective mobilization of stem/progenitor cells from the bone marrow in mice.²²

Although we did not observe differences in the number of stem/progenitor cells in the bone marrow of patients with ICMP, the functional capacity of BM-MNCs as determined by their colony-forming activity and their migratory response to SDF-1 was significantly reduced in BM-MNCs isolated from these patients. Intriguingly, the SDF-1-induced migration of the BM-MNCs and the colony-forming activity significantly correlated with the functional activity in vivo (Figure 3, a and b). Like their impaired migratory response to SDF-1 and their reduced colony-forming activity in vitro, injection of these BM-MNCs from patients with ICMP into mice with limb ischemia showed a significantly reduced ability to rescue limb perfusion after unilateral ligation of the femoral artery. We therefore conclude that the reduced functional activity rather than a decrease in a distinct stem/progenitor cell population is limiting the neovascularization

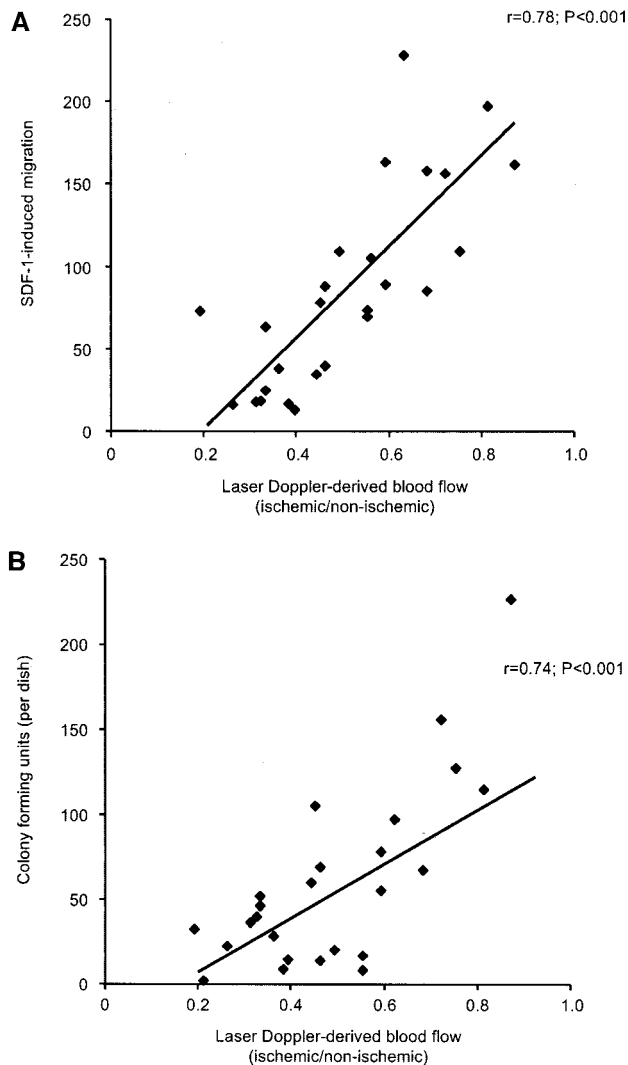


Figure 3. Correlation between functional activity of BM-MNCs in vivo as assessed by laser Doppler imaging and their in vitro cell characteristics. a, Correlation between neovascularization capacity of BM-MNCs and their migratory response to SDF-1 in vitro. b, Correlation between neovascularization capacity of BM-MNCs and their colony-forming capacity in vitro ($n=26$).

potential of BM-MNCs from patients with ICMP. However, we cannot rule out that the number of, for instance, side population cells or other not yet identified potent cell types may also be dysregulated in bone marrow of patients with ICMP. More studies are warranted for the identification of the specific cell type that is actually responsible for promoting neovascularization.

Migration is essential for stem/progenitor cells to invade the ischemic tissue. SDF-1 and VEGF are both profoundly upregulated in hypoxic tissue^{23,24} and may thus represent physiologically relevant chemoattractants for the recruitment of circulating progenitor cells to sites of ischemia. Indeed, intramuscular injection of the chemoattractant chemokine SDF-1 has recently been shown to increase the number of incorporated endothelial progenitor cells and to improve neovascularization in vivo.²⁵ Thus, the migratory response toward SDF-1 may indeed play a crucial role for integration

of progenitor cells in ischemic tissue. The exact mechanism underlying the impaired migratory response toward SDF-1 is unclear. It is reasonable to hypothesize that a downregulation of the receptor for SDF-1, CXCR4, may play a crucial role. However, our data indicate that the surface expression of CXCR4 in all studied cell populations does not differ between BM-MNCs from healthy controls and BM-MNCs from patients with ICMP. Therefore, alterations of intracellular pathways involved in the SDF-1-induced migration are more likely and deserve further investigation. In addition, we did not find a difference in the expression of CD49d (VLA-4), an important integrin on stem/progenitor cells mediating their interaction with endothelial cells via vascular cell adhesion molecule-1.

From our study population, it is difficult to conclude which may be the most important risk factor driving the functional differences in stem/progenitor cells between healthy controls and patients with ICMP. Patients with ICMP differed from healthy controls with respect to age, cardiovascular risk factors, presence of coronary heart disease, and left ventricular function. The extensive self-renewal capacity of hematopoietic stem cells implies that this cell population may not age and thus may provide undiminished replenishment of blood cells throughout the lifespan of an organism. In support of this idea, steady-state hematopoietic function is generally maintained in aged mice and humans.²⁶ When bone marrow from old mice is transplanted into irradiated young animals, so that the old bone marrow cells compete with young cells during replacement of the host's lymphohematopoietic tissues, old hematopoietic stem cells are not at a competitive disadvantage. However, more old than young hematopoietic stem cells are necessary to reconstitute the bone marrow of a recipient, most likely because of a reduced ability to home to and seed the bone marrow.²⁷ In our study population, the number of hematopoietic stem cells in the bone marrow showed no association with age. However, by univariate analysis, the functional capacity of the BM-MNCs as assessed by their in vivo neovascularization capacity showed a trend toward a reduction with age ($P=0.06$). A similar correlation was observed for age and the in vitro assessment of SDF-1-induced migration ($P=0.03$). In a multivariate model, however, other baseline characteristics were overriding this effect of age on the in vivo neovascularization capacity of the BM-MNCs (Table 2). These data suggest that ischemic heart disease and/or the presence of cardiovascular risk factors contribute significantly to the functional impairment of BM-MNCs in patients with ICMP. It is important to note, however, that the disparity in the baseline characteristics between the 2 patient groups limits the conclusions that can be drawn from the present study. Although it is reasonable to conclude that patients with ICMP have functionally impaired stem/progenitor cells with respect to their neovascularization capacity, the present study population of patients with ICMP itself is too homogeneous regarding left ventricular pump function, number of risk factors, and age to more definitively demonstrate which factor is indeed responsible for the functional impairment of BM-MNCs from patients with ICMP. Future studies including patients with normal left ventricular pump function and/or patients with impaired left

TABLE 2. Univariate and Multivariate Linear Regression Analysis of the Association Between Patients' Clinical Characteristics and In Vitro Parameters of Cell Function and the Neovascularization Capacity of Bone Marrow-Derived Mononuclear Cells as Measured by Recovery of Laser Doppler-Derived Relative Blood Flow in a Murine Model of Hindlimb Ischemia

| Variable | Univariate | | | | Multivariate | |
|-------------------------|--------------------------------------|--------|----------------------|-------|--------------------------------------|-------|
| | Patients and Healthy Controls (n=26) | | Patients Only (n=18) | | Patients and Healthy Controls (n=26) | |
| | r | P | r | P | r | P |
| Age | -0.38 | 0.064 | 0.260 | 0.33 | ... | ... |
| Sex | 0.032 | 0.87 | -0.165 | 0.49 | ... | ... |
| Hypertension | -0.646 | 0.001 | -0.204 | 0.43 | ... | ... |
| Hypercholesterolemia | -0.595 | 0.002 | 0.114 | 0.68 | ... | ... |
| Family history of CHD | -0.403 | 0.019 | 0.134 | 0.61 | ... | ... |
| Diabetes mellitus | -0.641 | 0.001 | -0.088 | 0.74 | ... | ... |
| No. of risk factors | -0.540 | 0.006 | 0.174 | 0.52 | 0.48±0.68 | 0.641 |
| NYHA classification | -0.630 | 0.001 | -0.116 | 0.66 | -2.62±4.53 | 0.79 |
| Ejection fraction (%) | 0.720 | 0.001 | 0.315 | 0.273 | 0.56±0.71 | 0.46 |
| Basal migration | 0.380 | 0.061 | 0.456 | 0.05 | ... | ... |
| SDF-1-induced migration | 0.781 | <0.001 | 0.634 | 0.004 | 3.07±1.95 | 0.008 |
| VEGF-induced migration | 0.352 | 0.078 | 0.374 | 0.13 | ... | ... |
| Colony forming capacity | 0.740 | <0.001 | 0.074 | 0.785 | 2.02±1.37 | 0.061 |

CHD indicates coronary heart disease.

ventricular function caused by dilated cardiomyopathy who lack risk factors for coronary heart disease will be needed to address this question.

Stem and progenitor cells may be involved not only in postnatal neovascularization but also in atherogenesis. A recent experimental study supports this novel concept that atherosclerosis progression is related to obsolescence of endogenous progenitor cells that normally repair and rejuvenate the arteries.²⁸ Specifically, Rauscher et al²⁸ demonstrated that chronic treatment with bone marrow-derived progenitor cells from young nonatherosclerotic apolipoprotein E-deficient (ApoE^{-/-}) mice prevents atherosclerosis progression in ApoE^{-/-} recipients despite persistent hypercholesterolemia. In contrast, treatment with bone marrow cells from older ApoE^{-/-} mice with atherosclerosis is much less effective, suggesting that progressive progenitor cell deficits in older animals may contribute to the development of atherosclerosis. The data of the present study support this novel concept of progressive stem/progenitor cell deficit in patients with ischemic heart disease. Mechanistically, however, from our data it appears more likely that this deficit is related to stem/progenitor cell dysfunction rather than stem/progenitor cell exhaustion.

The functional impairment of BM-MNCs from patients with ICMP may also limit their therapeutic potential for clinical cell therapy, especially when using an intravascular route of administration, which requires the progenitor cells to extravasate against a chemoattractant gradient to invade and home to ischemic tissue. Monitoring the migratory and colony-forming activity of stem/progenitor cells before cell therapy may serve as a surrogate test for identifying patients

who may derive greater benefit from cell therapy. Indeed, a recent subanalysis of the TOPCARE-AMI trial revealed that the migratory capacity of the infused cells determines the functional improvement of the treated patients.¹¹ Conversely, a recent experimental study suggests that fully functional bone marrow-derived endothelial progenitor cells from healthy donor mice restore the senescent host cardiac angiogenesis in a murine model of impaired neovascularization.²⁹ Thus, it is tempting to speculate that pharmacological or genetic manipulation of the functionally impaired BM-MNCs before reinfusion may improve their functional activity and subsequently may improve the patients' therapeutic benefit from cell therapy.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (Di 600/4-1 and Wa 1461/2-1) and the Alfred Krupp Stiftung. We thank Marion Muhly-Reinholz, Sylvia Rhiel, Tina Rasper, and Tino Röxe for their excellent technical assistance.

References

1. Towbin JA, Bowles NE. The failing heart. *Nature*. 2002;415:227-233.
2. Perin EC, Geng YJ, Willerson JT. Adult stem cell therapy in perspective. *Circulation*. 2003;107:935-938.
3. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430-436.
4. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A*. 2001;98:10344-10349.
5. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-705.
6. Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional

- function in pigs with chronic experimental myocardial ischemia. *J Am Coll Cardiol*. 2001;37:1726–1732.
7. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107:1395–1402.
 8. Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93–98.
 9. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
 10. Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation*. 2003;107:1024–1032.
 11. Britten M, Abolmaali ND, Assmus B, et al. Infarct remodeling following intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation*. 2003;108:2212–2218.
 12. Assmus B, Schachinger V, Teupe C, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009–3017.
 13. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002;106:1913–1918.
 14. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001;89:E1–E7.
 15. Schatteman GC, Hanlon HD, Jiao C, et al. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest*. 2000;106:571–578.
 16. Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation*. 2002;106:2781–2786.
 17. Deleted in proof.
 18. Deleted in proof.
 19. Fuchs S, Satler LF, Kornowski R, et al. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study. *J Am Coll Cardiol*. 2003;41:1721–1724.
 20. Perin EC, Dohmann HF, Borojevic R, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation*. 2003;107:2294–2302.
 21. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143–147.
 22. Aicher A, Heeschen C, Mildner-Rihm C, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003;9:1370–1376.
 23. Lee SH, Wolf PL, Escudero R, et al. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med*. 2000;342:626–633.
 24. Pillarisetti K, Gupta SK. Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1)1: SDF-1 alpha mRNA is selectively induced in rat model of myocardial infarction. *Inflammation*. 2001;25:293–300.
 25. Yamaguchi J, Kusano KF, Masuo O, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation*. 2003;107:1322–1328.
 26. Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. *Nat Immunol*. 2002;3:329–333.
 27. Morrison SJ, Wandycz AM, Akashi K, et al. The aging of hematopoietic stem cells. *Nat Med*. 1996;2:1011–1016.
 28. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, et al. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation*. 2003;108:457–463.
 29. Edelberg JM, Tang L, Hattori K, et al. Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. *Circ Res*. 2002;90:E89–E93.