Improvement of Endothelial Function by Systemic Transfusion of Vascular Progenitor Cells

Sven Wassmann, Nikos Werner, Thomas Czech, Georg Nickenig

Abstract—Endothelial dysfunction is characterized by abnormalities in vasoreactivity and is a marker of the extent of atherosclerosis. Cellular repair by circulating progenitor cells of ongoing vascular injury may be essential for vascular integrity and function and may limit abnormalities in vasoreactivity. Apolipoprotein E–deficient (apoE−/−) mice were splenectomized and treated with high-cholesterol diet for 5 weeks, resulting in marked impairment of endothelium-dependent vasodilation of aortic segments as compared with wild-type mice. Intravenous transfusion of 2×10^7 spleen-derived mononuclear cells (MNCs) isolated from wild-type mice on 3 consecutive days restored endothelium-dependent vasodilation in the apoE−/− mice, as measured 7, 14, and 45 days after transfusion. Histological analyses of aortic tissue identified fluorescent-labeled, exogenously applied progenitor cells that expressed the endothelial cell marker CD31 in the endothelial cell layer of atherosclerotic lesions. Progenitor cell treatment led to increased vascular nitric oxide synthase activity. Transfusion of either in vitro-differentiated Dil-Ac-LDL/lectin-positive endothelial progenitor cells, CD11b-positive (monocyte marker), CD45R-positive (B-cell marker), or Sca-1–positive (stem cell marker) MNC subpopulations significantly improved endothelium-dependent vasodilation, although these treatments were not as effective as transfusion of total MNCs. Depletion of MNCs of either CD11b-positive, CD45R-positive, or Sca-1–positive cells resulted in significant attenuation of endothelium-dependent vasodilation as compared with nondepleted MNCs; however, vasoreactivity was still significantly improved as compared with saline-treated apoE−/− mice. Intravenous transfusion of spleen-derived MNCs improves endothelium-dependent vasodilation in atherosclerotic apoE−/− mice, indicating an important role of circulating progenitor cells for the repair of ongoing vascular injury. More than 1 subpopulation of the MNC fraction seems to be involved in this effect. (Circ Res. 2006;99:e74-e83.)

Key Words: endothelial progenitor cells ■ apolipoprotein E–deficient mice ■ endothelial function ■ stem cells ■ nitric oxide

Endothelial dysfunction, which is manifested by abnormalities in vasoreactivity and inflammatory processes, is a marker of the extent of the atherosclerotic process and ongoing vascular injury. The presence of severely abnormal vasomotion is associated with a high incidence of cardiovascular events in patients with hypertension or coronary heart disease.1–4 On the molecular level, dysfunctional endothelium is caused by increased oxidative stress and reduced nitric oxide (NO) bioavailability.5 On the cellular level, endothelial dysfunction is associated with increased rates of endothelial cell apoptosis.6 In this context, it has been shown that levels of circulating apoptotic endothelial cells are elevated in patients with severe hypertension and acute coronary syndromes.7,8 This raises the possibility that endothelial dysfunction and atherosclerosis are, at least in part, based on an impaired regeneration of the damaged endothelium in the presence of ongoing vascular injury.

There is increasing evidence that regeneration of the heart and the vasculature is governed by circulating premature cells.9–11 One type of these cells, endothelial progenitor cells (EPCs), has been implemented in the myocardial repair after infarction and in the propagation of angiogenesis following ischemia.12–16 Furthermore, EPCs have been identified as important source of vascular repair after injury.17,18 We have recently demonstrated that EPCs can be isolated from spleen-derived mononuclear cells (MNCs) and that intravenous transfusion of spleen-derived MNCs and EPCs enhances reendothelialization and diminishes neointima formation after vascular injury.19

Therefore, we hypothesized that systemic application of exogenous vascular progenitor cells potentially enhances regeneration processes of the diseased endothelium and thereby limits abnormalities in vasoreactivity. We tested this notion in splenectomized apolipoprotein E–deficient (apoE−/−) mice fed cholesterol-rich diet, an animal model of premature atherosclerosis prone to develop endothelial dysfunction based on a severe lipid disorder.20,21

Materials and Methods

Materials
Oil red O solution, salts, and other chemicals were purchased from Sigma. L-012 was obtained from Wako Chemicals.

Original received June 11, 2004; resubmission July 13, 2006; revised resubmission September 1, 2006; accepted September 8, 2006.
From the Medizinische Klinik und Poliklinik II, Universitätsklinikum Bonn, Germany.
Correspondence to Dr Sven Wassmann, Medizinische Klinik und Poliklinik II, Universitätsklinikum Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. E-mail sven.wassmann@uni-bonn.de
© 2006 American Heart Association, Inc.
Circulation Research is available at http://circres.ahajournals.org
DOI: 10.1161/01.RES.0000246095.90247.d4

e74
Animals
Male, 12-week-old apoE−/− mice and C57BL/6J (wild-type) mice (Charles River, Sulzfeld, Germany) were used for this study. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. All mice were fed a high-fat, cholesterol-rich diet for 5 weeks, containing 21% fat, 19.5% casein, and 1.25% cholesterol (Sniff, Soest, Germany). Plasma lipid concentrations were determined by routine chemical methods. Low-density lipoprotein (LDL) cholesterol was determined using the Friedewald formula. The mice were killed after the indicated treatments and tissue samples and blood were collected immediately. All animal experiments were performed in accordance with institutional guidelines and the German animal protection law.

Splenectomy
Mice were anesthetized with 150 mg/kg body weight ketamine hydrochloride (Ketanest, Pharmacia) and 0.1 mg/kg body weight xylazine hydrochloride (Rompun 2%, Bayer). The spleen was dissected through a lateral incision of the left abdomen. Vessels were carefully ligated using 6/0 silk. After removal of the spleen, the abdomen was closed layer by layer with single sutures using 6/0 silk. Animals were allowed to recover for 7 days before further treatment was performed.

Preparation of MNCs
Spleens from wild-type mice were explanted and mechanically minced, and MNCs were isolated using a Picoll gradient (Lymphomite-M, Cedarlane). MNCs were stained with PKH-26, a fluorescent dye with long aliphatic tails for incorporation in the cell membrane that permits identification of these cells and their progeny, using the PKH26-GL Red Fluorescent Cell Linker Kit (Sigma) according to the instructions of the manufacturer. Labeled cells were counted and resuspended in 500 μL of normal saline solution for intravenous injection. In a subset of experiments, the same amount of spleen-derived MNCs was subjected to magnetic bead separation. MNCs were either separated into CD11b-positive (monocyte marker) or -negative cells, CD45R-positive (B-cell marker) or -negative cells, or Sca-1-positive (stem cell marker) or -negative cells. In brief, spleen-derived MNCs were washed, resuspended, and mixed with colloidal superparamagnetic microbeads conjugated to either monoclonal rat anti-mouse CD45R (B220), Sca-1, or CD11b (Mac-1) antibodies (MACS MicroBeads, Miltenyi Biotec). After incubation and additional washing, magnetic cell separation was performed by filling the cell suspension into a depletion column placed in the magnetic field of a magnetic bead separator (MACS Depletion Columns; MidiMACS Separator, Miltenyi). The collected effluent contained the negative MNC fraction depleted of either CD45R-, Sca-1−, or CD11b-positive cells. After taking the column out of the magnetic field, the attached CD45R-, Sca-1−, or CD11b-positive MNCs were collected in buffer. The separated MNC subpopulations were stained with PKH-26, counted, and resuspended in 500 μL of normal saline solution for intravenous injection.

Preparation of Spleen-Derived EPCs
Spleen-derived MNCs were seeded on fibronectin-coated 24-well plates in 0.5 mL of endothelial basal medium (EBM) (CellSystems). MNCs were either used directly or the same amount of spleen-derived MNCs was subjected to further processing before transfection, resulting in 1.5×10^5 CD11b-negative MNCs, 5×10^5 CD11b-positive MNCs, 1×10^5 CD45R-negative MNCs, 1×10^5 CD45R-positive MNCs, 1×10^5 Sca-1-negative MNCs, 3×10^5 Sca-1-positive MNCs, and 6×10^5 in vitro–differentiated EPCs. In addition, 2×10^5 cells per day of spleen-derived MNCs from 12-week-old apoE−/− wild-type mice received a corresponding amount of normal saline solution without cells. After cell/saline treatment, all mice were fed normal chow, until aortas were excised 7, 14, and 45 days after the last transfection, respectively. The treatment protocol is depicted in Figure 1.

Transfusion Regimen
At the end of the 5-week treatment period with cholesterol-rich diet, spleenectomized apoE−/− mice received PKH-26–stained, spleen-derived cells by intravenous tail vein injection on 3 consecutive days. Spleen-derived MNCs (2×10^6 cells per day) from wild-type mice were either used directly or the same amount of spleen-derived MNCs was subjected to further processing before transfection, resulting in 1.5×10^5 CD11b-negative MNCs, 5×10^5 CD11b-positive MNCs, 1×10^5 CD45R-negative MNCs, 1×10^5 CD45R-positive MNCs, 1×10^5 Sca-1-negative MNCs, 3×10^5 Sca-1-positive MNCs, and 6×10^5 in vitro–differentiated EPCs. In addition, 2×10^5 cells per day of spleen-derived MNCs from 12-week-old apoE−/− mice fed with normal chow were used. Control animals (apoE−/− wild-type mice) received a corresponding amount of normal saline solution without cells. After cell/saline treatment, all mice were fed normal chow, until aortas were excised 7, 14, and 45 days after the last transfusion, respectively. The treatment protocol is depicted in Figure 1.

Measurement of Vascular Reactive Oxygen Species
Reactive oxygen species (ROS) release in intact aortic segments was determined in organ baths filled with oxygenated modified Tyrode buffer (37°C), as previously described.22 Adventitial tissue was carefully removed, and 3-mm segments of the thoracic aorta were investigated. A resting tension of 10 mN was maintained throughout the experiment. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: 20 and 40 mmol/L KCl, 1 nmol/L to 10 μmol/L phenylephrine, 10 nmol/L to 100 μmol/L carbachol (assessment of endothelium-dependent vasodilation after precontraction with phenylephrine), and 1 nmol/L to 10 μmol/L nitroglycerin (assessment of endothelium-independent vasodilation after precontraction with phenylephrine). The drug concentration was increased when vasodilatation or -relaxation was completed. Drugs were washed out before the next substance was added.

NOS Activity Assay
Vascular NOS activity was quantified by measuring the conversion of [14C]arginine to [14C]citrulline in aortic homogenates using a nitric oxide synthase assay kit (Calbiochem), as previously described.24 In brief, excised aortic segments were immersed in ice-cold homogenization buffer that contained 250 mmol/L Tris/HCl (pH 7.4), 10 mmol/L EDTA, and 10 mmol/L EGTA and were mechanically homogenized. NOS activity was determined in 10 μg of protein aliquots of the aortic homogenates after the addition of [14C]arginine, NADPH, tetrahydrobiopterin, and CaCl2. Rat cerebellum extracts, containing elevated amounts of neuronal NOS, were used as positive controls, whereas aortic lysates incubated in the presence of N0-L-arginine methyl ester (L-NAME) served as blanks. The amount of [14C]citrulline was quantified with a β-counter (Beckman).

Histological Analysis
Aortic segments were embedded in Tissue Tek OCT embedding medium (Miles), snap frozen, and stored at −80°C. For immunohistochemical analysis, samples were sectioned on a Leica cryostat (7 μm) and placed on poly-L-lysine (Sigma) coated slides. Cryosections were assessed for PKH-26–positive cells and for the endothelial cell marker CD31 (platelet endothelial cell adhesion molecule-1

Wassmann et al Vascular Progenitor Cells and Endothelial Function e75
cholesterol, HDL, and LDL. Male, 12-week-old apoE

Endothelial Function in ApoE

Effect of Transfusion of Spleen-Derived MNCs on

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed using the ANOVA test followed by the Neuman–Keuls post hoc analysis. P<0.05 indicates statistical significance.

Results

Effect of Transfusion of Spleen-Derived MNCs on Endothelial Function in ApoE−/− Mice

Male, 12-week-old apoE−/− mice and age-matched wild-type mice were splenectomized and fed a high-fat, high-cholesterol diet for 5 weeks. Total cholesterol, HDL, and LDL cholesterol plasma concentrations were significantly elevated in apoE−/− animals compared with wild-type mice (total cholesterol, 802±55 versus 111±11 mg/dL, P<0.05, versus wild-type; HDL cholesterol, 267±29 versus 78±11 mg/dL, P<0.05, versus wild-type; LDL cholesterol, 520±58 versus 19±2 mg/dL, P<0.05, versus wild-type; triglycerides, 76±13 versus 80±8 mg/dL). Figure 2A shows that in contrast to wild-type mice, endothelium-dependent vasodilation was profoundly impaired in apoE−/− mice, indicating endothelial dysfunction, as assessed in aortic ring preparations in response to increasing concentrations of carbachol (maximal relaxation: 53.5±2.7% versus 92.2±5.7%, P<0.05, versus wild-type). Endothelium-independent vasorelaxation induced by nitroglycerin was similar between the groups (Figure 2B).

After splenectomy and treatment with high-cholesterol diet for 5 weeks, apoE−/− mice received 3 transfusions of spleen-derived MNCs from wild-type mice (2×10^7 cells per day) or normal saline solution without cells (control) intravenously on 3 consecutive days. Aortic tissue was obtained 7, 14, and 45 days after the last transfusion, respectively.

[PECAM-1]) (monoclonal rat anti-mouse CD31 antibody, clone MEC13.3, BD Pharmingen) with an indirect immunofluorescent method. Cryosections were postfixed in 95% ethanol for 5 minutes. Slides were preincubated with normal horse serum (Vector Laboratories) for 30 minutes each. The primary antibody was applied for 1 hour at room temperature. Slides were then incubated with a biotin-conjugated secondary antibody (goat anti-rat, BD Pharmingen) for 30 minutes, followed by application of fluorescein isothiocyanate (FITC)-labeled Streptavidin for 30 minutes. Nuclear staining was performed using 4’,6-diamidino-2-phenylindole (DAPI) (Linaris). Isotype-specific antibodies (Santa Cruz Biotechnology) were used for negative controls. Sections were washed and mounted with fluorescent mounting medium (Dako) for fluorescent microscopic analysis. For the detection of atherosclerotic lesions, aortic segments were sectioned on the cryostat and sections were fixed with 3.7% formaldehyde for 1 hour, rinsed with deionized water, stained with oil red O working solution (0.5%) for 30 minutes, and rinsed again. Hematoxylin staining was performed according to standard protocols. All sections were examined under a Nikon E600 microscope using Lucia Measurement Version 4.6 software. For quantification of atherosclerotic plaque formation in the aortic root, lipid-staining area and total area of serial histological sections were measured. Atherosclerosis data are expressed as lipid-staining area in percentage of total surface area. The investigator who performed the histological analyses was blinded to the treatment of the respective animal group.

[Image 140x526 to 464x726]
apoE diet for 5 weeks. As displayed in Figure 2A, transfusion of 14-day MNCs, 87.1 normalized 14 days after MNC transfusion (maximal relax-
thelium-dependent vasodilation was still almost completely
the last transfusion, respectively. Figure 3 shows that endo-
function of aortic segments was assessed 14 and 45 days after
results were not as pronounced as 7 and 14 days
MNCs, indicating a stable effect on endothelial function, although the effect was not as pronounced as 7 and 14 days
MNCs from wild-type animals (2 \times 10^7 cells per day) were
endothelium-dependent vasodilation was still completely
endothelium-independent vasodilation induced by carbachol (A) and endothelium-independent vasore-
taxation induced by nitroglycerin (B), both expressed as percentage of maximal phenylephrine-induced vasoconstriction. Mean ± SEM, n = 4 to 8 per group with 4 rings per animal. *P < 0.05 vs apoE−/−, #P < 0.05 vs wild-type MNCs.

in splenectomized apoE−/− mice treated with high-cholesterol
diet for 5 weeks. As displayed in Figure 2A, transfusion of apoE−/−-derived MNCs improved endothelium-dependent va-
sodilation after 7 days but was less effective than transfusion of MNCs obtained from wild-type animals (maximal relaxation: 75.0 ± 3.4%; P < 0.05 versus apoE−/− control and versus wild-type MNCs). Endothelium-independent vasorelaxation was not altered (Figure 2B).

Figure 2. Effect of intravenous treatment with spleen-derived MNCs on endothelial function in apoE−/− mice. Twelve-week-old apoE−/− mice and wild-type (WT) mice were splenectomized and treated with high-fat, high-cholesterol diet for 5 weeks. apoE−/− mice received 3 transfusions of spleen-derived MNCs from wild-type mice (WT-MNC) or apoE−/− mice fed normal chow (apoE-MNC) or cell-free saline (apoE) intravenously on 3 consecutive days. Endothelial function of isolated aortic segments was assessed 7 days after the last transfusion in organ
chamber experiments. Endothelium-dependent vasodilation induced by carbachol (A) and endothelium-independent vasorelaxation induced by nitroglycerin (B), both expressed as percentage of maximal phenylephrine-induced vasoconstriction. Mean ± SEM, n = 4 to 8 per group with 4 rings per animal. *P < 0.05 vs apoE−/−, #P < 0.05 vs wild-type MNCs.

Duration of the Effect of Transfusion of Spleen-Derived MNCs on Endothelial Function
After splenectomy and treatment with high-cholesterol diet for 5 weeks, apoE−/− mice received 3 transfusions of spleen-
derived MNCs from wild-type mice (2 \times 10^7 cells per day) or cell-free saline intravenously on 3 consecutive days. To
investigate the duration of the transfusion effect, endothelial function of aortic segments was assessed 14 and 45 days after
the last transfusion, respectively. Figure 3 shows that endo-
thelium-dependent vasodilation was still almost completely
normalized 14 days after MNC transfusion (maximal relaxation: 14-day MNCs, 87.1 ± 3.3%; P < 0.05 versus apoE−/−
control). Endothelium-dependent vasodilation was still sig-
ificantly improved 45 days after the last transfusion of
MNCs, indicating a stable effect on endothelial function, although the effect was not as pronounced as 7 and 14 days
after MNC transfusion (maximal relaxation: 74.2 ± 2.9%; P < 0.05 versus apoE−/− control and versus 14-day MNCs)
(Figure 3). Endothelium-independent vasorelaxation was not impaired as compared with wild-type mice and was not
affected by MNC treatment after 14 and 45 days, respectively (data not shown).

Effect of Transfusion of Spleen-Derived MNC Subgroups on Endothelial Function
To investigate which MNC subtype mediates the improve-
ment of endothelial function in apoE−/− mice, spleen-derived
MNCs from wild-type animals (2 \times 10^7 cells per day) were
subjected to further processing before transfusion.
First, to explore the role of vascular premature cells, spleen-derived MNCs from wild-type mice (2 \times 10^7 cells per
day) were differentiated in vitro into EPCs. Transfusion of this cell population resulted in significant improvement of
endothelium-dependent vasodilation after 7 days but was not
as effective as transfusion of complete MNCs before differen-
tiation into EPCs (maximal relaxation: 72.0 ± 2.8%; P < 0.05 versus apoE−/− control and versus undifferentiated
MNCs) (Figure 4A). Endothelium-independent vasorelaxation was not altered (data not shown).

Second, CD11b-positive (monocyte marker), Sca-1–positive (stem cell marker), and CD45R-positive (B-cell marker)
MNCs were obtained by magnetic bead separation. Separate
transfusion of these cell preparations significantly improved
endothelium-dependent vasodilation after 7 days, although
these treatments were not as effective as the transfusion of the
complete MNC population (maximal relaxation: saline con-
trol, 40.1 ± 4.7%; CD11b-positive MNCs, 73.7 ± 1.8%; CD45R-positive MNCs, 65.7 ± 2.2%; Sca-1–positive MNCs, 56.2 ± 2.6%; P < 0.05 versus apoE−/− control and versus complete MNCs) (Figure 4A). Endothelium-independent va-
morelaxation was not altered (data not shown).
Third, spleen-derived MNCs were depleted of either CD11b-positive, Sca-1-positive, or CD45R-positive cells by magnetic bead separation. Separate transfusion of these depleted MNC preparations resulted in a significantly attenuated effect on endothelium-dependent vasodilation after 7 days as compared with transfusion of nondepleted MNCs; however, endothelial function was still significantly improved as compared with saline-treated apoE−/− mice (maximal relaxation: CD11b-negative MNCs, 64.0±3.9%; CD45R-negative MNCs, 71.9±3.0%; Sca-1-negative MNCs, 68.1±2.2%; P<0.05 vs apoE−/− control and versus nondepleted MNCs) (Figure 4B).

Histological Analysis of Aortic Tissue
Treatment of apoE−/− mice with high-fat, high-cholesterol diet for 5 weeks resulted in atherosclerotic lesion formation in the aortic root and thoracic aorta, as demonstrated by oil red O staining (Figure 5A and 5B). Before intravenous transfusion, MNCs and EPCs were stained with the fluorescent dye PKH-26, allowing identification of these cells in the vessel wall. After transfusion, PKH-26-positive cells were located mainly in aortic atherosclerotic plaque area but not in the liver or lungs. Immunohistological analysis of PKH-26-positive cells attached to the luminal side of the plaque revealed that these cells expressed CD31 (PECAM-1), indicating the expression of an endothelial cell-specific marker in the transfused cells after homing within the endothelial cell layer. In addition, these cells showed typical histoanatomical features of endothelial cells in the microscopic high-magnification analyses of 7-μm-thin cryosections. PKH-26/CD31 double-positive cells were found in the endothelial cell layer after transfusion of EPCs (Figure 5C through 5F) as well as of MNCs (Figure 5G through 5J). In addition, PKH-26/CD31 double-positive cells were also identified in the endothelial cell layer after transfusion of CD11b-positive
MNCs, but not after transfusion of CD45R-positive MNCs (data not shown).

**Vascular Activity of NOS**

Increased NO bioavailability attributable to enhanced endothelial cell-mediated NO production after progenitor cell transfusion may account for the improvement of endothelium-dependent vasodilation. Therefore, NO synthase (NOS) activity was assessed in aortic tissue from apoE−/−/H11002/H11002 mice that received cell-free saline or transfusion of wild-type MNCs or EPCs. Figure 6A demonstrates that aortic NOS activity was significantly increased 7 and 14 days after treatment with wild-type MNCs (167.6±16.1% and 200.7±28.3% of saline-treated apoE−/−/H11002/H11002, respectively; *P<0.05 versus apoE−/−/H11002/H11002). Treatment with in vitro–differentiated EPCs also resulted in significant enhancement of NOS activity, although the effect was not as pronounced as compared with wild-type MNCs (153.3±24.6% of saline-treated apoE−/−, *P<0.05 versus apoE−/−).

**Vascular Production of ROS**

Decreased vascular oxidative stress may contribute to increased NO bioavailability. Therefore, vascular ROS production was assessed in aortic segments of apoE−/− mice after treatment with cell-free saline or progenitor cells by L-012 chemiluminescence assays. Figure 6B shows that aortic ROS production was not significantly influenced by treatment with MNCs or EPCs.

**Effect of Transfusion of Spleen-Derived MNCs on Atherosclerotic Plaque Formation in apoE−/− Mice**

After splenectomy and treatment with high-cholesterol diet for 5 weeks, apoE−/− mice received 3 transfusions of spleen-derived MNCs from wild-type mice or cell-free saline intravenously on 3 consecutive days. Atherosclerotic plaque formation in the aortic root was assessed 45 days after the last transfusion by histomorphometric analyses of oil red O–stained cryosections. A, Representative sections of the aortic root. B, Quantification of atherosclerotic plaque formation, expressed as lipid-staining area in percentage of total surface area. Mean±SEM, n=4 to 5 per group.

Discussion

We and others have recently shown that progenitor cells derived from spleen homogenates and in vitro–differentiated EPCs drive the process of reendothelialization after local vascular injury.17,18 These findings established the notion that, rather than local endothelial cells, circulating...
progenitor cells play an important role in regeneration processes of the endothelium and may therefore be an important target for therapeutic intervention. Interestingly, increasing the number of circulating EPCs by statin, estrogen, or granulocyte colony-stimulating factor (G-CSF) treatment, physical exercise, or cell transfusion not only accelerated reendothelialization but also profoundly inhibited neointima formation after vascular injury.17–19,25–27 Studies in patients with in-stent restenosis after percutaneous coronary intervention demonstrating reduced numbers and impaired adhesion of circulating EPCs and novel approaches with EPC-capturing coronary stents, leading to enhanced reendothelialization and diminished restenosis point toward an important role of EPCs for the inhibition of restenosis in a clinical setting.28–30 To extend these observations, it is crucial to show that application of EPCs and related cell populations also interferes with the more disseminated vascular disease of atherosclerosis as opposed to the described effects after focal vascular injury. This is of relevance because neointima formation after vascular injury only in part resembles the pathology of atherosclerosis, and surgical injury models can only crudely correlate to the situation in humans. Therefore, additional experiments in models that display typical characteristics of atherosclerosis, such as the apoE−/− mouse,20 are warranted. These mice develop endothelial dysfunction, as characterized by abnormal vasoreactivity, reflecting the extent of atherosclerosis.

A recently published study has shown that chronic treatment of young apoE−/− mice with bone marrow–derived progenitor cells from young nonatherosclerotic apoE−/− mice or wild-type mice prevented the development of atherosclerotic lesions.31 Treatment was started at an age of 3 weeks and repeated every 2 weeks in the presence of a cholesterol-rich diet. However, this does not resemble the situation in humans, who usually present in the clinic with established forms of atherosclerosis. In contrast to the aforementioned study, increased atherosclerotic plaque size and altered plaque composition in apoE−/− mice treated intravenously with EPCs or bone marrow cells was demonstrated in one study, whereas in another study no effect on atherosclerotic plaque size and structure was demonstrated in nonischemic apoE−/− mice treated intravenously with bone marrow MNCs, but an increased lesion size was seen in the apoE−/− group with hindlimb ischemia.32,33 In the present study, we observed no significant effect of MNC treatment on the extent of atherosclerotic lesion formation in animals with already established atherosclerosis. However, atherosclerotic plaque area in the aortic root was increased by approximately 17% after MNC treatment as compared with saline treatment. The relevance of this nonsignificant trend in this small group of investigated animals is not clear at present. These contradictory findings may possibly result from different methodological approaches in the apoE−/− mice, and the true nature of the contribution of EPCs and MNCs to atherosclerotic plaque development remains unclear in this animal model of atherosclerosis. However, a prospective clinical study demonstrated that low numbers and impaired function of circulating EPCs were associated with increased cardiovascular mortality in patients with coronary artery disease,34 supporting a protective role of EPCs in the atherosclerotic process in humans.

In the present study, we treated 17-week-old apoE−/− mice that had already developed endothelial dysfunction and atherosclerotic plaque formation attributable to the preceding cholesterol-rich diet. Intravenous application of MNCs as well as of EPC and MNC subpopulations improved endothelial-dependent vasodilation, suggesting that this cell-based treatment can limit abnormalities in vasoreactivity associated with atherosclerotic plaque development. Of note, the improvement of endothelial function sustained for at least 45 days after cell therapy. Cell treatment with MNCs and EPCs was associated with enhanced vascular NOS activity, which finally results in increased NO production. It is conceivable that this effect is an important mechanism underlying the observed improvement of vascular function, because increased NO concentrations together with unaltered vascular ROS production result in enhanced NO bioavailability, which leads to improvement of endothelium-dependent vasodilation.3 In addition, NO was shown to differentially regulate proliferation of EPCs and to promote EPC differentiation into mature endothelial cells, which may serve as an important autocrine/paracrine effect after EPC homing.35 The results of our study are in agreement with clinical studies that showed a positive correlation between EPC counts in vitro and endothelium-dependent vasodilatation of the forearm36 and demonstrated improvement of endothelium-dependent vasorelaxation of the leg after bone marrow MNC implantation in patients with limb ischemia.37

The histological analyses after cell treatment revealed that the exogenously administered MNCs and EPCs incorporated into the vessel wall predominantly in aortic atherosclerotic plaque areas. Localization of the transfused cells within the endothelial cell monolayer, presence of histoanatomical characteristics of endothelial cells, and expression of the endothelial cell marker CD31 suggest that these initially premature cells turned into mature endothelial cells by differentiation or fusion processes and subsequently contributed to improvement of endothelium-dependent vasoreactivity by direct and/or paracrine effects. The fact that endothelium-dependent vasodilation was still markedly improved 45 days after cell application demonstrates that the transduction of progenitor cells induced a prolonged effect on endothelial function and suggests a stable homing of the cells within the vessel wall. This is an important finding contrasting the results of a recent study that demonstrated only transient engraftment (<28 days) of bone marrow–derived hematopoietic stem cells in ischemic myocardium.38

Atherosclerosis in humans is often associated with abnormalities of both endothelium-dependent and -independent vasoreactivity. In contrast to endothelium-dependent vasodilation, we found no alteration of endothelium-independent vasorelaxation in our model of apoE−/− mice. Furthermore, we observed no effect of treatment with progenitor cells on the latter parameter. Data concerning endothelium-independent vasodilation at baseline and after experimental intervention are inconsistent in hypercholesterolemic mouse models, which may be caused by differences in the specific mouse models and strains, diet, duration of treatment, and
model and protocol of assessment of vascular function. In addition, there are differences in human and rodent atherosclerosis. In agreement with animal data, impairment of endothelium-independent vasorelaxation is not found in all clinical studies assessing endothelial function in human atherosclerotic subjects. A trial investigating endothelial function in patients with limb ischemia demonstrated a selective improvement of endothelium-dependent vasodilation, but not of endothelium-independent vasodilation after MNC treatment.\(^{37}\) In a study by Hill and colleagues, a weak correlation of EPC number and endothelium-independent vasoreactivity was found; however, only subjects with a high ratio of flow-mediated to nitroglycerin-induced brachial reactivity had higher EPC counts than did subjects with a low ratio.\(^{36}\) However, it cannot be excluded that treatment with premature cells improves endothelium-dependent vasodilation without affecting endothelium-independent abnormalities in vasoreactivity in atherosclerotic humans.

It remains incompletely understood from the data of our study which exact cell type of the spleen-derived MNCs is responsible for the observed beneficial effect on endothelium-dependent vasoreactivity. EPCs are good candidates because these cells obviously can differentiate into mature endothelial cells. The results of our study support an important role of this cell type, because in vitro–differentiated EPCs markedly improved endothelium-dependent vasodilation. Mouse EPCs are partially characterized by the expression of the stem cell marker Sca-1. Consistently, transfusion of Sca-1–positive cells led to an improvement of endothelium-dependent vasodilation in the present study. However, transfusion of MNCs that were depleted of Sca-1–positive cells resulted in an attenuation but not complete inhibition of the beneficial effect on endothelial function, indicating that this premature cell population is not the only cell population mediating the improvement of endothelium-dependent vasoreactivity. It was recently shown that endothelial (progenitor) cells could be differentiated from monocytic cell types, and in addition, it was reported that B cells could decrease atherosclerotic disease progression in apoE\(^{-/-}\) mice.\(^{39–42}\) Therefore, we also explored the effect of MNC subpopulations that were positive or negative of the CD11b (monocyte marker) or CD45R (B-cell marker) surface antigens. CD11b-positive and CD45R-negative MNCs and that EPC phenotype after differentiation was important for the functional neovascularization capacity as compared with freshly isolated, nondifferentiated CD14-positive MNCs.\(^{43}\) In our study, both CD11b-positive and -negative MNC subpopulations had an effect on endothelium-dependent vasoreactivity. Interestingly, we found CD31-positive, exogenously applied, PKH-26–positive cells in the endothelial layer after transfusion of CD11b-positive MNCs but not of CD45R-positive MNCs. It may be speculated that the special composition of spleen-derived MNCs, including monocytes, B cells, EPCs, and possibly other (premature) cells, allows important interactions that could lead to a more efficient homing and differentiation of these cells via autocrine and paracrine mechanisms and finally to an improved function of mature endothelial cells. The results of our study suggest that more than 1 subpopulation of the MNC fraction is needed to exert the described beneficial effect on endothelial function. On the other hand, other cell types may differentiate into endothelial (progenitor) cells, and increased transdifferentiation rates as well as enhanced fusion may account for the pronounced effects on endothelium-dependent vasoreactivity. Further studies will have to clarify the cellular and molecular features which determine the successful homing and differentiation of premature cells, to maximize the therapeutic potential of this cell-based treatment.

Application of MNCs isolated from age-matched apoE\(^{-/-}\) mice fed normal chow was less efficient in the enhancement of endothelium-dependent vasodilation as compared with the transfusion of wild-type MNCs. Therefore, it may be assumed that the inherited lipid disorder seems to be of importance for the beneficial effects of premature cells, although the underlying mechanisms remain obscure. It is improbable that the effect relates to a potential influence of wild-type cells on lipid metabolism, because lipid levels were not affected by cell treatment and the transfused cells were almost exclusively detected within vascular lesion sites and were not found in other organs such as the liver.

The presented data reveal that severe abnormalities in endothelium-dependent vasoreactivity can be limited by progenitor cell treatment in atherosclerotic mice. Our results establish another intriguing role of progenitor cells besides myocardial repair after infarction, angiogenesis, and vascular repair after local injury. Further studies are warranted to elucidate the detailed molecular and cellular mechanisms involved in this regenerative process. It remains to be determined whether the findings of the present study can be translated to human disease. In our study, a high number of premature cells was obtained from spleens and transfused intravenously on 3 consecutive days. This approach was used to gain direct mechanistic insight into the regenerative potential of progenitor cells in disseminated atherosclerotic disease in an animal model of human disease, which is, of course, not feasible in humans. However, the results of our model show that this premature cell-based approach has a profound regenerative potential on endothelial function in atherosclerotic disease and demonstrate a rather stable effect on endothelium-dependent vasoreactivity as long as 45 days. It is not clear how long lasting the treatment effect is in ongoing disseminated vascular injury during hypercholesterolemia.

\(\text{Wassmann et al} \quad \text{Vascular Progenitor Cells and Endothelial Function} \quad e81\)
whether repeated treatments could maintain the beneficial effect, and whether the effect on endothelium-dependent vasodilation may be counterbalanced by the lack of effect on endothelium-independent vasoreactivity in atherosclerotic humans. The prospective Endothelial Progenitor Cells in Coronary Artery Disease study supports the protective role of high numbers of circulating progenitor cells in the atherosclerotic process in humans. As a perspective, enhanced endogenous mobilization, increased homing, and improved function of vascular progenitor cells after medical treatment may be options to possibly transfer progenitor cell-based therapeutic approaches in humans to improve vascular function in organs endangered by atherosclerosis.

Acknowledgments
The excellent technical assistance of Sybille Richter, Simone Jäger, Isabel Faez-Maletz, Bianca Klöckner, Susanne Schnell, Annika Bohner, and Kathrin Paul is greatly appreciated.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft and a research grant from the University of the Saarland, Homburg/Saar, Germany.

Disclosures
None.

References
Improvement of Endothelial Function by Systemic Transfusion of Vascular Progenitor Cells
Sven Wassmann, Nikos Werner, Thomas Czech and Georg Nickenig

Circ Res. 2006;99:E74-E83; originally published online September 21, 2006;
doi: 10.1161/01.RES.0000246095.90247.d4
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/99/8/E74

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://ww.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/