Bone Marrow–Derived Progenitor Cells Modulate Vascular Reendothelialization and Neointimal Formation

Effect of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibition

Nikos Werner,* Josef Priller,* Ulrich Laufs, Matthias Endres, Michael Böhm, Ulrich Dirnagl, Georg Nickenig

Objective—Atherosclerosis and restenosis after vascular injury are both characterized by endothelial dysfunction, apoptosis, inappropriate endothelialization, and neointimal formation. Bone marrow–derived endothelial progenitor cells have been implicated in neovascularization, resulting in adult blood vessel formation. Despite the anticipated stem cell plasticity, the role of bone marrow–derived endothelial progenitor cells has not been clarified in vascular lesion development.

Methods and Results—We investigated vascular lesion formation in mice after transplantation of bone marrow transfected by means of retrovirus with enhanced green fluorescent protein. Carotid artery injury was induced, resulting in neointimal formation. Fluorescence microscopy and immunohistological analysis revealed that bone marrow–derived progenitor cells are involved in reendothelialization of the vascular lesions. Treatment with rosuvastatin (20 mg/kg body wt per day), a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, enhanced the circulating pool of endothelial progenitor cells, propagated the advent of bone marrow–derived endothelial cells in the injured vessel wall, and, thereby, accelerated reendothelialization and significantly decreased neointimal formation.

Conclusions—Vascular lesion development initiated by endothelial cell damage is moderated by bone marrow–derived progenitor cells. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibition promotes bone marrow–dependent reendothelialization and diminishes vascular lesion development. These findings may help to establish novel pathophysiological concepts and therapeutic strategies in the treatment of various cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2002;22:1567-1572.)

Key Words: endothelium □ endothelial progenitor cells □ vascular injury □ neointima □ 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition

Atherosclerosis leads to serious medical conditions, including coronary heart disease, the main cause of death in the Western world.1 Atherosclerotic lesions are initiated by endothelial cell damage, followed by macrophage adhesion and invasion as well as smooth muscle cell migration and growth.2 If stenosis due to atherosclerosis is treated by balloon angioplasty and/or stent implantation, restenosis of the target vessel is frequently observed.3 This is caused by endothelial cell damage and rapid neointimal formation, which is mainly composed of proliferating smooth muscle cells.4,5 Thus, despite numerous epidemiological, cellular, and molecular differences, atherosclerosis and restenosis after vascular injury share at least 1 important pathophysiological step: endothelial cell damage followed by impaired reendothelialization.6,6 It is currently believed that this reendothelialization is controlled by the adjacent endothelial cells within the vessel wall.7

Accumulating evidence indicates that bone marrow–derived cells are involved in repair processes throughout the cardiovascular system.8–11 Accordingly, endothelial progenitor cells (EPCs) have been implicated in neovascularization in the context of peripheral arterial disease as well as coronary heart disease.8,12–16 In addition, bone marrow–derived cells may help to reestablish myocardial tissue after myocardial infarction.10,15,17–21

Little is known about the relevance of EPCs in the development and cure of atherosclerotic lesions. It is unclear whether bone marrow–derived cells are involved in the reendothelialization and neointimal formation causing restenosis after vascular injury and whether these events can be...
modulated by 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, which are thought to influence the release of progenitor cells from the bone marrow.22,23

In the present study, mice with green fluorescent protein (GFP)-marked bone marrow were subjected to carotid artery injury to elucidate the role of the bone marrow in reendothelialization and neointimal formation. Furthermore, the effect of rosuvastatin versus placebo on circulating EPCs and reendothelialization was evaluated.

Methods

Retroviral Gene Transfer and Bone Marrow Transplantation

Reconstitution of hematopoiesis with GFP-marked bone marrow cells was performed as described previously.24,25 Briefly, bone marrow was harvested from 8- to 10-week-old male C57BL/6 mice 2 days after treatment with 5-fluorouracil (Sigma Chemical Co). Bone marrow cells were stimulated in DMEM/15% FCS supplemented with 20 ng/mL recombinant murine interleukin-3, 50 ng/mL human interleukin-6 (PromoCell), and 50 ng/mL rat stem cell factor (generously provided by Amsen. Munich, Germany) for 48 hours. Subsequently, bone marrow cells were cocultured with irradiated (13-Gy) viral producer cells. The ecotropic packaging cell line GP+E86 was used for the generation of MGIrl22Y retroviral vector particles. The MGIrl22Y vector was generated by cloning the enhanced GFP (Clontech) gene upstream from the internal ribosomal entry site from the encephalomyocarditis virus linked to a mutant dihydrofolate reductase gene (L22Y) in a murine stem cell virus vector. The presence of recombinant retrovirus was excluded in assays that used a muI dunni test cell line. After 48 hours of coculture, nonadherent bone marrow cells were rinsed off the producer cell monolayer. Transduced bone marrow cells (5 × 10⁶) were transplanted by tail vein injection into lethally irradiated 8- to 10-week-old male C57BL/6 mice (11-Gy cumulative dose). Flow cytometric analysis of GFP expression was performed in peripheral blood leukocytes of mice 8 months after transplantation of GFP-expressing bone marrow cells and in leukocytes of control mice that did not receive GFP-labeled bone marrow. Approximately 70% of white blood cells were stably expressing the fluorophore in GFP–bone marrow chimeras. All myeloid and lymphocytic populations were labeled, but the levels of GFP expression varied within lineages. The highest expression was observed in monocytes/macrophages and granulocytes.

Carotid Injury and Treatment

Six months after bone marrow reconstitution, the mice received daily doses of rosuvastatin (Crestor, generously provided by AstraZeneca, Cheshire, UK) at 20 mg/kg body wt subcutaneously. Control animals received a corresponding amount of normal saline. Treatment was started 10 days before carotid injury and was continued until tissue harvesting. Carotid artery injury was induced as described previously.26 Briefly, the mice were anesthetized with halothane/N₂O. By use of a dissecting microscope (MZ6, Leica), the bifurcation of the left carotid artery was exposed via a midline incision of the ventral side of the neck. Two ligatures were placed proximally and distally around the external carotid artery. The distal ligature was then tied off. After temporary occlusion of the internal and common carotid artery with ligatures, a transverse arteriotomy was performed between the ligatures of the external carotid artery to introduce a wire, the proximal ligature of the external carotid artery was tied off. Restoration of normal blood flow was confirmed, and the skin was closed with single sutures with the use of 6/0 silk. Animals were allowed to recover, and carotid arteries were harvested after 14 days. All animal procedures were approved and were in accordance with the institutional guidelines.

Vessel Harvesting

After perfusion-fixation with 4% paraformaldehyde, carotid arteries were embedded in tissue Tek OCT embedding medium (Miles Laboratories), snap-frozen, and stored at −80°C. Samples were sectioned on a Leica cryostat (7 μm) and placed on poly-l-lysine (Sigma)–coated slides for immunohistochemical analysis.

Histocchemistry

Cyrosctions were assessed for endothelial cell markers (von Willebrand factor [vWF], polyclonal antibody, clone A 0082, Dako; vascular endothelial growth factor receptor-2 [VEGF-R2], clone A3, Santa Cruz; and vascular endothelial cadherin, F-8, Santa Cruz), smooth muscle cells (mAb smooth muscle α-actin, clone 1A4, Sigma), and inflammatory cell markers (mAb Mac-3, clone M3/84; mAb CD45.1, clone A20; Pharmingen) with a direct immunofluorescence method. For light microscopy, a peroxidase-conjugated anti-GFP antibody (mAb GFP, B-2, Santa Cruz) with DAB staining (Dako) or APAAP Mouse Monoclonal IgG (F-8, Santa Cruz) with BCIP/NBT (Dako) was used. Nuclear staining was performed with the use of 4′,6-diamidino-2-phenylindole (Dapi, Dainis). Isotype-specific secondary antibodies (Santa Cruz) were used for negative controls. For immunohistochemistry, tissue cryosections were postfixed in 4% formaldehyde for 2 minutes. Slides were preincubated with 0.5% Igepal (Sigma) and 5% normal goat serum (Sigma) for 30 minutes each. The primary antibody was applied for 2 to 3 hours at room temperature or at 4°C overnight, followed by application of the appropriate TRITC-conjugated secondary antibody (Sigma) for 30 minutes. Sections were washed and mounted with fluorescent mounting medium (Dako) for fluorescence microscopic analysis. For morphometric analyses, hematoxylin and eosin staining was performed according to standard protocols. All sections were examined under a Nikon E600 microscope. For morphometric analyses, Lucia Measurement Version 4.6 software (Nikon, Germany) was used to measure external elastic lamina, internal elastic lamina, and lumen circumference, as well as medial and neointimal area of 25 sections per animal. To determine reendothelialization, mice received 50 μL of 5% Evans blue diluted in normal saline by tail vein injection 10 minutes before euthanasia, followed by perfusion-fixation. Arteries were opened longitudinally and placed between slides with mounting medium (Dako). After transparency scanning (Nikon SMZ8000), stained carotid arteries were planimetered (Lucia Measurement Version 4.6 software), and the ratio between the area stained in blue and the total carotid area was calculated.

Fluorescence-Activated Cell Sorter Analysis

Peripheral blood of rosuvastatin-treated or placebo (normal saline)–treated mice was collected at 1, 10, 11, and 24 days after the initiation of treatment. After lysis of whole blood and Fc blockade (Fc-Block, Pharmingen), the viable lymphocyte population was analyzed for the expression of Sca-1-FITC (clone E13-161.7, Pharmingen) and vascular endothelial growth factor receptor-2 (clone A3, Santa Cruz) conjugated with the corresponding phycoerythrin-labeled secondary antibody (Sigma). Isotype-identical antibodies served as controls (Becton Dickinson). Single- and 2-color flow cytometric analyses were performed by using a Becton Dickinson FACScan equipped with an argon ion laser. Data were evaluated by Cellquest software.

Cell Culture

Spleens were mechanically minced, and mononuclear cells were isolated by use of a Ficoll (Biochrom) gradient. Cells (4 × 10⁶) were seeded into fibronectin (Sigma)–coated 24-well plates in 0.5 mL endothelial basal medium (CellSystems) and FITC–labeled lectin (UEA-1, Sigma). At least 3 high-power fields of each
well were analyzed. Cells positive for Dil-Ac-LDL and lectin staining were judged to be EPCs and were counted.

Statistical Analysis
Results are presented as mean±SEM. Significance of the difference between 2 measurements was determined by unpaired Student t test, and multiple comparisons were evaluated by the Newman-Keuls multiple comparison test. Values of P<0.05 were considered significant.

Results
Carotid Injury Model
Fourteen days after carotid injury, vessels were harvested and subjected to histological analysis. Figure 1 shows a representative example of highly reproducible neointimal formation in an artery after injury compared with an unaltered carotid artery.

Retrovirus-Mediated Transfer of the GFP Gene Into Hematopoietic Cells
Transduction efficiency into murine myeloid clonogenic progenitors averaged 80% to 90%. Retrovirally transduced hematopoietic colonies expressed high levels of GFP, as determined by direct visualization with phase-contrast and fluorescence microscopy. All myeloid and lymphocytic lineages displayed a 50% to 80% range of GFP labeling, and GFP expression was stable during the entire observation period.

Reendothelialization From the Bone Marrow
Mice with reconstituted GFP bone marrow were subjected to carotid artery injury procedures. Fourteen days after injury, harvested vessels were analyzed by direct fluorescence microscopy to visualize GFP-positive cells. Immunohistochemical localization of antigens in the arterial vasculature was complicated by the presence of complex molecules such as collagen, elastin, cholesterol, and fluorescent lipids that exhibit autofluorescence over a wide spectrum of wavelengths. Figure 2A and 2B shows the contralateral uninjured carotid artery, in which an autofluorescent signal from the elastic fibers but not from GFP-positive cells was detected. In contrast, scanning of the injured artery revealed GFP-positive cells predominantly at the endothelial monolayer (Figure 2C). Light-microscopic control stainings with a peroxidase-conjugated antibody against GFP confirmed that these cells express the GFP protein and were therefore derived from the bone marrow (data not shown). To confirm the commitment of these GFP-positive cells to the endothelial cell lineage, immunohistochemical analysis with monoclonal antibodies against vWF (Figure 2D) and vascular endothelial cadherin (data not shown) were used, demonstrating that luminal GFP-positive cells represent endothelial cells. Control stainings with Dapi (data not shown) show the spindle-shaped nucleus of endothelial cells.

The concomitant appearance of GFP and endothelial cell lineage marker indicates that reendothelialization is influenced by bone marrow–derived cells.

The neointima-forming tissue is mainly composed of vascular smooth muscle cells. Direct fluorescence microscopy and additional staining with antibodies against α-actin (data not shown) revealed that smooth muscle cells within the analyzed vessel segments were not GFP positive and therefore did not stem from the bone marrow. Macrophages and leukocytes were immunohistochemically detected with antibodies against CD45 and Mac-3. However, these cells were primarily localized in the connective tissue adjacent to the injured vessel wall and only rarely at the luminal surface (please see online Figure I, available at www.ahajournals.org).

Effect of Rosuvastatin Treatment on EPCs
Mice were treated with rosuvastatin (20 mg/kg body wt per day). Peripheral blood collected after 1, 10, 11, and 24 days was submitted to fluorescence-activated cell sorter analysis to quantify Sca-1/vascular endothelial growth factor receptor 2–positive cells. Representative fluorescence-activated cell sorter analyses of the control group and of the rosuvastatin-treated group are shown in online Figure IIA and IIB (available at www.ahajournals.org).

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Figure 1. Hematoxylin and eosin (HE) staining of mouse carotid artery. A, Uninjured carotid artery (control). B, Prominent neointimal formation 14 days after arterial injury.
mononuclear cells were seeded in each well. After an in vitro expansion period of 7 days, DiI-Ac-LDL–positive and lectin-positive endothelial cells were quantified as depicted in Figure 3B. A 1-day treatment with rosuvastatin caused an increase to 12,090 endothelial cells (179±34% control) compared with placebo treatment (5995 cells). Further increases were ascertained after 10 and 11 days; however, the enhancing effect of rosuvastatin was more sustained, with a maximum of 54,380 cells after 24 days (454±72% control).

Rosuvastatin Enhances Reendothelialization From the Bone Marrow
Mice were treated with either placebo or rosuvastatin (20 mg/kg body wt per day) for 10 days before carotid injury. Vessels were harvested 14 days later and evaluated. Rosuvastatin treatment profoundly increased the reendothelialization process, as shown in Figure 4A and 4B. GFP- and vWF-positive cells were abundant at the luminal side of the lesion, indicating accelerated reendothelialization. A semi-quantitative analysis of GFP- and vWF-positive cells in relation to the total number of GFP-negative endothelial cells indicated a significant increase in bone marrow–derived endothelial cells committed to reendothelialization after vascular injury (Figure 5A). Thus, the rosuvastatin-induced increase in EPCs is associated with an increased arrival and attachment of bone marrow–derived cells at the site of injury and accelerates endothelial repair mechanisms.

Rosuvastatin Decreases Neointimal Formation
Because rapid reendothelialization is thought to inhibit neointimal formation,26 we investigated whether the observed rosuvastatin-induced increase of bone marrow–derived endothelial reconstitution results in increased reendothelialization and diminished neointimal formation. Figure 5B demonstrates accelerated reendothelialization in animals after a 7-day treatment with rosuvastatin compared with placebo-treated animals. Rosuvastatin therapy almost completely prevented the development of intimal hyperplasia, suggesting that the bone marrow–driven rapid reendothelialization with rosuvastatin was associated with reduced neointimal formation (Figure 5C and 5D).

Discussion
An intact endothelial monolayer is essential for the physiological functioning of the vasculature.27 Metabolic conditions, such as hypercholesterolemia and diabetes, cause endothelial dysfunction. Mechanical manipulations, such as angioplasty, lead to endothelial denudation.28 Endothelial dysfunction and denudation are both associated with neointimal formation, resulting in narrowing and, ultimately, in occlusion of the diseased vessel.5,6 Improvement of endothelial function and accelerated reendothelialization reduce neointimal formation.5,27 This is of special interest regarding the prevention of restenosis after angioplasty.29 In the past, repair of endothelial cell damage was thought to rely on the outspreading of cells from the adjacent intact vascular wall.7 Recent studies have raised the possibility that bone marrow–derived cells contribute to neoangiogenesis after vascular occlusion and possibly to myocardial regeneration after infarction. In addition, circulating bone marrow–derived cells...
resembling immature vascular smooth muscle cells seem to contribute to neointimal formation after severe damage of the vessel wall. Therefore, stem cell–based intervention could be the basis of novel treatment methods. Despite these intriguing findings, little is known about the role of the bone marrow in the setting of reendothelialization. Our data indicate that bone marrow–derived cells are directed to vascular lesion sites to reestablish an intact endothelial layer. These cells, characterized by surface markers of immaturity, circulate in the peripheral blood and are capable of adhering to the injured vascular wall. Obviously, the physiological numbers and features of these cells are insufficient to overcome exogenously applied vessel damage, because the unwanted and deleterious neointimal formation occurs reproducibly. Therefore, it may be beneficial to increase the numbers and enhance the attachment of bone marrow–derived progenitor cells to accelerate reendothelialization. Vascularly derived growth factors as well as HMG CoA reductase inhibitors (statins) have been shown to increase the number of circulating premature endothelial cells in mice and humans. Interestingly, the pool of EPCs seems to be dependent on coronary risk factors and coronary heart disease, with decreasing numbers in the peripheral blood associated with an increased risk profile. Statins have been shown to produce a decrease in cardiovascular event rates, an effect that is often assumed to be related to their lipid-lowering properties. However, there is evidence that so-called pleiotropic effects, independent of lipid lowering, could account, at least partially, for some of the beneficial clinical effects. In the case of enhanced release of EPCs, the activation of phosphatidylinositol 3-kinase–dependent and Akt-dependent pathways seem to be involved. In the mouse model in the present study, rosuvastatin treatment not only enhanced the number of circulating EPCs but also profoundly enhanced reendothelialization due to attachment of circulating bone marrow–derived cells. This novel finding clearly extends the presently established knowledge regarding bone marrow–derived vascular cells and the reendothelialization processes. Moreover, it suggests that the known advantages of statin treatment may be partially caused by a beneficial influence on bone marrow–derived progenitor cells. However, several questions remain unanswered and warrant further investigation: How are EPCs released in greater quantities by statin treatment? Is this release caused by enhanced proliferation and differentiation of committed stem cells within the bone marrow, or is it due to an increased release of a preexisting pool? What are the exact mechanisms governing these events? What makes endothelial cells attach to the injured vessel wall? Are there inducible adhesion molecules at the site of injury, or are there integrin-like
structures on the endothelial cells, or do both occur, propagating the interaction of endothelial cells with the vascular wall? How is this modulated by statins?

These results provide novel insight into the biology of vascular lesion repair. In contrast to widely believed dogma, repair of endothelial cell damage is modulated not only by adjacent vessel structures but also by bone marrow–derived cells. As well as adding another facet to the properties of HMG CoA reductase inhibitors, this discovery raises the possibility that treatment regimens involving bone marrow–derived cells might help prevent advanced vascular lesion formation.

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References

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**Figure legends**

**Figure I** Inflammatory cells in the connective tissue adjacent to the arterial carotid injury. 

- **a:** Bone marrow-derived GFP positive cells (green), 10x. 
- **b:** Mac-3 positive macrophages were only occasionally found at the luminal side but mostly in the connective tissue, 10x. 
- **c:** corresponding overlay, 10x. 
- **d, g:** Bone marrow-derived GFP positive cells (green). 
- **e:** CD45 positive leucocytes (red) and 
- **h:** Mac-3 positive macrophages (red) in surrounding connective tissue with corresponding overlay (**f, i**).

**Figure II** Increase of endothelial progenitor cells after rosuvastatin treatment. 

Representative FACS analyses of control (**a**) and after administration of rosuvastatin (**b**). Isotype-identical antibodies served as controls (Becton Dickinson).