Role of Bone Marrow-Derived Progenitor Cells in Cuff-Induced Vascular Injury in Mice

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Objectives—Arterial injury results in vascular remodeling associated with proliferation and migration of smooth muscle cells (SMCs) and the development of intimal hyperplasia, which is a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions. However, the origin of SMCs and other cells in the development of vascular remodeling is not yet fully understood.

Methods and Results—We utilized a cuff-induced vascular injury model after transplantation of the bone marrow (BM) from green fluorescent protein (GFP)-transgenic mice. We found that macrophages were major cells recruited to the adventitia of the vascular injury lesion along with SMCs and endothelial cells (ECs). While investigating whether those cells are derived from the donor, we found that most of the macrophages were GFP-positive, and some of the SMCs and ECs were also GFP-positive. Administration of the anti-c-fms antibody resulted in a marked decrease in macrophages and a relative increase of SMCs, while administration of antibodies against the platelet-derived growth factor receptor-β caused a prominent decrease in SMCs and a relative increase in macrophages.

Conclusions—The current study indicates that BM-derived cells play an important role in vascular injury, and that the differentiation of macrophages and SMCs might be dependent on each other. (Arterioscler Thromb Vasc Biol. 2004; 24:1-7.)

Key Words: macrophage ■ smooth muscle cell ■ endothelial cell ■ vascular injury ■ bone marrow

Arterial injury results in proliferation and migration of smooth muscle cells (SMCs) and the development of intimal hyperplasia, a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions. However, the origin of SMCs, which engage in the development of neointimal thickening during vascular disease, is not yet fully understood. One possibility is that medial SMCs are phenotypically modified and migrate into the intima, where they proliferate and secrete extracellular matrix components. It has also been proposed that adventitial fibroblasts move into the neointima and give rise to cells with smooth-muscle–like properties.

Recently, several groups have reported that cells of recipient origin take part in the formation of neointimal SMCs during the development of transplant vasculopathy. These results agree with the notion that adult bone marrow (BM) contains multipotent cells that can develop into various lineages. It has also been shown that endothelial progenitor cells (EPCs) can transdifferentiate into SMCs. Thus, the origin of SMCs in atherosclerotic lesions is a source of controversy, and it is important to understand the contribution of BM-derived cells to neointimal formation in vascular pathology.

In vascular injury or remodeling, it is not clear whether one specific type of multipotent precursor cell can differentiate into endothelial cells (ECs), SMCs, or macrophages, or whether there are different precursor cells for each cell lineage. We have reported that administration of anti-c-fms antibody can prevent early atherosclerosis in apolipoprotein E-deficient (apoE−/−) mice. We have also shown that administration of antibodies against the platelet-derived growth factor receptor-β (anti-PDGF-β) can prevent the recruitment of SMCs, but not of macrophages in the atherosclerotic lesions in apoE−/− mice. These results indicate the important role of macrophages in the initiation of the lesion and recruitment of SMCs in hyperlipidemia-induced atherosclerosis. However, it is not known whether the recruitment of macrophages is critical for the migration of SMCs in vascular injury.

Therefore, we have two goals in this study. One is to explore the contribution of BM-derived cells to the development of vascular remodeling. The other is to examine whether blocking the cell differentiation by a specific antibody can affect the lesion formation in vascular injury. For this purpose we have utilized an inflammation-dependent vascular disease
model induced by polyethylene cuff placement around the femoral artery after BM transplantation (BMT) from green fluorescent protein (GFP)-transgenic mice.

Methods

Mice

All experimental protocols were performed in accordance with the guidelines of Kyoto University, Japan. GFP-transgenic mice with C57BL/6 background were a generous gift from Dr. M. Okabe (Osaka University, Japan). The mice were kept in a temperature-controlled facility on a 14-hour light/10-hour dark cycle, with free access to food and water. Mice were fed a normal chow diet containing 8.7% (wt/wt) fat and 0.063% (wt/wt) cholesterol (Oriental Yeast, Chiba, Japan) for the entire period of the experiment.

Bone Marrow Transplantation

Femurs of male or female, 8- to 12-week-old GFP-transgenic mice were dissected, and surrounding muscle tissue was removed by microscissors. Bones were then left in Dulbecco’s modified Eagle’s medium (DMEM). Both ends of the bones were cut with scissors, and the marrow was flushed with DMEM using a syringe with a 21-gauge needle. The marrow clusters were disaggregated by vigorous pipetting. BM cells were washed, resuspended in PBS, and counted. Eight-week-old female C57BL/6 mice were subjected to a lethal dose of total body irradiation (9 Gy) using the Gammacell 40 Exactor Irradiator (Nordion International). Each irradiated recipient received 5 × 10^5 BM cells extracted from GFP-transgenic mice in 0.5 mL PBS by tail vein injection. Mice used for BMT experiments were housed in sterilized cages and fed sterilized normal chow diet.

Cuff Placement

Mice were anesthetized with barbiturate complex [propylene glycol 17.9% (v/v), ethanol 8.9% (v/v), sodium 5-ethyl-5-(1-methylbutyl) barbiturate 10.7% (v/v)]. The right femoral artery was dissected from its surroundings. A nonconstrictive polyethylene cuff (PE50, 0.58 mm inner diameter, 0.965 mm outer diameter, 2 mm length; Becton Dickinson) was placed loosely around the right femoral artery.

Antibody Administration

AFS98, a rat monoclonal anti-murine c-fms antibody (IgG2a), which inhibits colony formation dependent on macrophage-colony stimulating factor (M-CSF) and cell growth by blocking the binding of M-CSF to its receptor c-fms, was previously described as an anti–c-fms antibody.8 APB5, a rat monoclonal anti-murine anti-PDGFR-β antibody (IgG2a), which blocks the PDGFR-β-mediated signaling pathway, was also described.9 Four C57BL/6 mice in each group were administered 1 mg of AFS98, APB5, or isotype-matched irrelevant rat IgG (y2A) once a day for 2 weeks after cuff placement. Eight-week-old female C57BL/6 mice were subjected to cuff placement. After 4 weeks of BMT, the recipient mice were phlebotomized, and the recipient mice were transfused with 0.1% hydrochloric acid. Four weeks after BMT, the recipient mice were phlebotomized, and the circulating leukocytes were then checked for the expression of GFP by flow cytometry. Cuff placement was performed at least 4 weeks after BMT. Cuff placement, the lumen of the cuffed artery was more restricted after cuff placement (Figure 1A, 1B, and 1D). Although we found that the accumulating in the lesion were GFP-positive (Figure 1A and 1B), suggesting that those cells were derived from the donor BM. In contrast, in the sham-operated artery, GFP-positive cells were hardly detected (Figure 1C). We found that more than 85% of the cells were positive for GFP (data not shown); this finding indicates that most of the leukocytes were derived from the donor BM. One or two weeks after cuff placement, cuffed or sham-operated femoral arteries were examined under fluorescence microscopy. In the cuffed artery, the majority of the cells accumulating in the lesion were GFP-positive (Figure 1A and 1B), suggesting that those cells were derived from the donor BM. In contrast, in the sham-operated artery, GFP-positive cells were hardly detected (Figure 1C). We found that the accumulation of BM-derived cells in the vascular remodeling lesion was significantly increased from 1 week to 2 weeks after cuff placement (Figure 1A, 1B, and 1D). Although we did not find a visible change in intimal thickening after cuff placement, the lumen of the cuffed artery was more restricted than that of the sham-operated artery (Figure 1E).

Results

Recruitment of Bone-Marrow–Derived Progenitor Cells in Cuff-Induced Vascular Remodeling

To elucidate the involvement of BM-derived cells in cuff-induced vascular remodeling lesions, BM cells from GFP-transgenic mice were transplanted into lethally irradiated C57BL/6 mice before cuff placement. After 4 weeks of BMT, we confirmed the reconstitution of the hematopoietic system by checking the fluorescence of blood leukocytes by flow cytometry. We found that more than 85% of the cells were positive for GFP (data not shown); this finding indicates that most of the leukocytes were derived from the donor BM. One or two weeks after cuff placement, cuffed or sham-operated femoral arteries were examined under fluorescence microscopy. In the cuffed artery, the majority of the cells accumulating in the lesion were GFP-positive (Figure 1A and 1B), suggesting that those cells were derived from the donor BM. In contrast, in the sham-operated artery, GFP-positive cells were hardly detected (Figure 1C). We found that the accumulation of BM-derived cells in the vascular remodeling lesion was significantly increased from 1 week to 2 weeks after cuff placement (Figure 1A, 1B, and 1D). Although we did not find a visible change in intimal thickening after cuff placement, the lumen of the cuffed artery was more restricted than that of the sham-operated artery (Figure 1E).

Macrophages are the Major Component in the Cuff-Induced Vascular Remodeling Lesion

Next, to examine the recruitment of macrophages in the cuffed lesion, we stained the tissue with BM8. We found many cells recruited to the adventitia of the cuffed artery, most of which were positive for BM8 (Figure 2A), indicating the role of monocyte-macrophages in vascular remodeling.
lesions. In the sham-operated femoral artery, we found few BM8-positive cells (Figure 2B).

BM Cells Can Differentiate into Vascular Smooth Muscle Cells

To examine whether BM-derived cells can differentiate into SMCs in the vascular remodeling lesion, we stained the tissue with Cy3-labeled anti-SMA (clone 1A4) and anti-SM1 (clone KM995) antibodies. We found a number of 1A4- and KM995-positive cells in the adventitia of the lesion (Figure 3B and 3E). With the colorization of GFP signals, we observed that some of the 1A4- and KM995-positive cells were also positive for GFP (Figure 3C and 3F), indicating that BM-derived cells can also differentiate to SMCs in the cuff-induced vascular remodeling lesion. However, in the earlier time point at 1 week after cuff placement, we could find few SMCs in vascular remodeling lesion (Figure 3H).

Interference Exists Between Macrophages and Smooth Muscle Cells

To examine whether inhibiting the differentiation to macrophage or SMC by mAb could affect the manner of accumulation and differentiation of BM-derived cells in the vascular remodeling lesion, we administered an antagonistic rat mAb against murine c-fms (M-CSF receptor) (clone AFS98) or PDGFR-β (clone APB5) to C57BL/6 female mice which had undergone cuff placement. In comparison with the lesion from mice administered with control IgG (clone 2A) (Figure 4C), we found that the treatment with AFS98 caused a marked decrease in macrophages in the lesion (Figure 4A and 4G). Interestingly, the density of SMCs was inversely increased (Figure 4D and 4H) in response to this treatment. In contrast, administration of APB5 resulted in a marked increase in macrophages (Figure 4B and 4G) with a concomi-

Figure 1. A through C, Representative microscopic photographs of BM-derived GFP-positive cells in C57BL/6 mouse vascular remodeling lesion. Four weeks after BMT, a nonconstrictive polyethylene cuff was placed around the right femoral artery in four mice in each group. The cuffed (A, 1 week after cuff placement; B, 2 weeks after cuff placement) or sham-operated (C) femoral arteries were examined under fluorescence microscopy. D and E, Quantitative analyses of BM-derived cell area (D) and femoral artery lumen area (E) after cuff placement showed a significant difference between 2 groups. Data from 20 slices per mouse artery are shown as mean±SEM. *P<0.05, **P<0.01. Scale bars: 100 μm.

Figure 2. Numerous macrophage-like cells accumulating in the cuff-induced vascular remodeling lesion. After 2 weeks of cuff placement as described in Figure 1, tissues were subjected to immunohistochemistry with biotinylated anti-mouse macrophage antibody BM8. A number of cells were BM8-positive cells in cuffed femoral artery (A), but in the sham operated femoral artery, those cells could hardly be found (B). Scale bars: 100 μm.
tant decrease of SMCs (Figure 4E and 4H), suggesting that a certain interaction occurs between macrophages and SMCs during the vascular remodeling process.

To estimate the effects of anti–PDGFR-β or anti–c-fms mAb on vascular remodeling, we measured the lumen size of the artery treated with the two kinds of mAb and γ2A. We

Figure 3. BM-derived SMCs in C57BL/6 mouse vascular remodeling lesion. After the same procedure in Figure 1, tissues were subjected to immunohistochemistry with antibodies to Cy3-labeled SMA (B, H) or SM1 (E). A, D, and G are fluorescent microscopic photographs for GFP. G, H, and I are fluorescent microscopic photographs from femoral artery of 1 week after cuff placement. All the others are samples at 2 weeks after cuff placement. C, F, and I are merged images of GFP and Cy3 signal from A and B, D and E, and G and H, respectively. Scale bars: 100 μm

Figure 4. Progenitors of SMC and macrophage have opposite roles in the lesion formation. A total of 12 C57BL/6 mice (8 weeks of age) were injected for 2 weeks with 1 mg of AFS98 (n=4), APB5 (n=4), or γ2A (n=4) every day after cuff placement. Each mouse was euthanized and the femoral artery was subjected to immunohistochemistry with anti-macrophage antibody (A, B, and C) or anti-SMA antibody (D, E, and F). A and D are from mice injected with AFS98, B and E are from mice injected with APB5, and C and F are from mice given γ2A. Ratio of the number of macrophages (G) and SMCs (H) to whole vascular remodeling lesion area had a significant difference in each group. Data from 20 slices per mouse are shown as mean±SEM. *P<0.05, **P<0.01. Scale bars: 100 μm
found no distinct difference in the lumen size of the femoral artery through administration of AFS98, APB5, or γ2A (data not shown).

We also examined whether each antibody administration had any effect on tissue formation after cuff placement. The calculated vascular remodeling lesion area of each mouse treated with AFS98, APB5, and γ2A was $1.18 \times 10^4 \pm 5.38 \times 10^3 \, \mu m^2$, $1.43 \times 10^5 \pm 7.27 \times 10^4 \, \mu m^2$, and $1.82 \times 10^4 \pm 1.11 \times 10^4 \, \mu m^2$, respectively (mean±SEM of 20 slices from each of 4 mice, P<0.05 versus γ2A). Less tissue formation was observed in the mice treated with AFS98 and APB5 than in mice treated with γ2A. This result indicates that AFS98 and APB5 administration could inhibit tissue formation after cuff placement. Further, to examine whether APB5 or AFS98 has an effect on BM-derived cell incorporation, we performed cuff placement and administered each antibody to mice that had been subjected to BMT. By measuring BM-derived cells accumulating in the cuff-induced lesion, we found a significant decrease of GFP-positive cells by mAb administration (data not shown), indicating that APB5 and AFS98 also affected the incorporation of BM-derived cells.

Endothelial Progenitor Cells Are Recruited to the Cuffed Vascular Remodeling Lesion

Because it is not known whether EPCs can contribute to cuff-induced vascular remodeling lesion formation in the injured femoral artery, we performed a series of endothelial staining. We found that the endothelial lining of the intima was clearly stained with anti-CD31 antibody, and that small vessels in the adventitia were also stained. There were also some CD31-positive cells clustered outside the small vessels in the adventitia in the cuffed lesions (Figure 5A), but not in the sham-operated lesions (Figure 5B). Because CD31 can also be expressed on monocyte-macrophages, we stained the tissue with anti-vWF antibody, another EC-specific marker, and compared the expression with GFP-positive cells. As shown in Figure 6E, the endothelial lining of the intima and small vessels in the adventitia were also positive for vWF. Some of the clustered cells in the adventitia were positive for vWF and GFP, while the endothelial lining of the intima of the artery and small vessels in the adventitia were only positive for vWF (Figure 6F), indicating the involvement of angiogenesis from vasa vasorum. Notably, as we observed that significantly fewer SMCs could be found 1 week after cuffing (Figure 3B), EPCs could scarcely be found in the vascular remodeling lesion at this earlier phase (Figure 6C).

Discussion

In this study, we have clearly shown that BM-derived cells are critically involved in the lesion formation of cuff-induced vascular remodeling in mice. In this setting, BM-derived macrophages, SMCs, and ECs contributed to the lesion formation.
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apoE induce intimal thickening of the cuffed artery when we used the technique of the cuff placement, because we were able to not been able to reproduce their results. This may be due to ening in the cuff-induced vascular injury model, 12,13 we have macrophages and SMCs during the lesion formation. These results suggest an interaction between macrophages and SMCs during the lesion formation.

Although previous investigators have shown intimal thickening in the cuff-induced vascular injury model,12,13 we have not been able to reproduce their results. This may be due to the technique of the cuff placement, because we were able to induce intimal thickening of the cuffed artery when we used apoE–/− mice fed with high-fat diet (unpublished observation, Xu et al). Indeed, we found a marked inflammatory change in the adventitial region around the cuffed artery. However, little is known about inflammatory responses in the adventitia after vascular injury, and adventitial and perivascular reactions are largely ignored. Recent clinical and experimental data by other investigators suggest that constrictive vascular remodeling is in large part responsible for lumen loss associated with restenosis.14,15 Scott et al have indicated that the adventitia may be important in the first wave of growth after angioplasty of coronary arteries, with later growth of the lesion occurring in the neointima.16 Therefore, studying the mechanism of cell recruitment to the adventitia in the vascular remodeling region is important for the understanding of the pathogenesis of restenosis.

Recent studies for transplant atherosclerosis have demonstrated that most of the neointimal α-actin–positive SMCs in recipient coronary arteries or aortas were from host origin,43 suggesting that these SMCs might be at least in part from BM-derived smooth muscle progenitor cells. In this study, we have demonstrated that at least three types of cells, macrophage, SMC, and EC are recruited from the BM to the adventitia of the cuff-induced vascular injury site. The characteristic feature of those cells is to form a cluster in the lesion. However, we have not determined when and how those cells migrate to the adventitia. Therefore, it is very important to understand the timing and pathway of cell migration in the pathogenesis of vascular injury. Elucidating the involvement of soluble factors in this model, such as chemokines and adhesion molecules, would also be intriguing.

In this study we have shown that administration of anti–c–fms antibody inhibited the recruitment of macrophages, and increased the recruitment of SMCs to the vascular injury lesion in wild-type mice. This finding is different from our report on apoE–/− mice, where we showed that the antibody inhibited the recruitment of SMCs as well as macrophages in early atherosclerotic lesion.4 Therefore, in hyperlipidemia-induced atherosclerosis, the recruitment of monocyte-macrophage is prerequisite for the migration of SMCs for the lesion formation; this paradigm was not applied to the current vascular injury model. If the common progenitors for macrophage and SMC exist, our data might indicate that BM-derived cells are playing an important role in vascular injury, but not in hyperlipidemia-induced atherosclerosis. The result with anti–PDGFR-β is also different from our previous observation in apoE–/− mice,9 in which the antibody to apoE–/− mice failed to affect the density of macrophages in advanced atheroma- tous lesions. It was also notable that administration of anti–PDGFR-β increased the recruitment of macrophages in this study. Thus, in the vascular injury model, blocking the differentiation of one cell type can increase the recruitment or differentiation of the other cell type. Although we have not determined whether the progenitors of macrophages and SMCs are derived from the same precursor cell, anti–c–fms or anti–PDGFR-β might affect the differentiation of common precursor cells.

Schmeisser et al reported that BM-derived macrophages might contribute to neovascularization by in situ transdifferentiation to EC-like cells.17 We found that in the vascular injury lesion there were many cells positive for CD31, which is an endothelial marker and is also positive for monocyte-lineage. However, vWF-positive cells were much smaller in number in this lesion. Furthermore, most of the cells forming a small vessel were positive for vWF, but negative for GFP, indicating that the source of the ECs forming a small vessel in the adventitia is from vasa vasorum, not from the BM.

Terada et al18 and Ying et al19 demonstrated that embryonic stem cells can spontaneously fuse with mononuclear BM cells20 or brain cells in vitro to form pluripotent tetraploid hybrids. In this study, there are a number of BM-derived cells generated after cuff placement in the cuff-induced vascular remodeling lesion. These BM-derived cells play an important role for lesion formation. Those two reports showed that the frequency of cell fusion was very low (2×10−6 to 10−4), although it is difficult to directly correlate the in vitro findings of embryonic stem cells to our in vivo study. It is possible that some of the BM-derived positive cells in our experiments resulted from fusion between BM cells and vascular cells; however, this phenomenon would be an unlikely explanation for the extent of BM involvement seen in this study.

In summary, we have provided evidence that BM-derived cells are playing a critical role in cuff-induced vascular injury in mice. Understanding the interaction among the cells involved in the lesion formation will be important for regulating the accumulation of inflammatory cells in the vascular injury lesion.

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