Physiological pathway of differentiation of hematopoietic stem cell population into mural cells

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Endothelial cells (ECs), which are a major component of blood vessels, have been reported to develop in adulthood from hematopoietic cell populations, especially those of the monocyte lineage. Here we show that mural cells (MCs), another component of blood vessels, develop physiologically during embryogenesis from a hematopoietic stem cell (HSC) population, based on the in vitro culture of HSCs and histological examination of acute myeloid leukemia 1 mutant embryos, which lack HSCs. As in the embryo, HSCs in adult bone marrow differentiate into $CD45^+CD11b^+$ cells before differentiating into MCs. Moreover, $CD45^+CD11b^+$ cells are composed of two populations, $CD11b^{high}$ and $CD11b^{low}$ cells, both of which can differentiate into MCs as well as ECs. Interestingly, in a murine ischemia model, MCs and ECs derived from the $CD11b^{low}$ population had a long-term potential to contribute to the formation of newly developed blood vessels in vivo compared with the $CD11^{high}$ population, which could not. Moreover, injection of the $CD11b^{high}$ population induced leaky blood vessels, but the $CD11b^{low}$ population did not. With respect to the permeability of vessels, we found that angiopoietin 1, which is a ligand for Tie2 receptor tyrosine kinase expressed on ECs and is suggested to induce cell adhesion between ECs and MCs, is produced by the $CD11b^{low}$ population and plays a critical role in the formation of nonleaky vessels. These observations suggested that the $CD11^{low}$ cell population serves as a good source of cells for in vivo blood vessel regeneration.

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Abbreviations used: ALP, alkaline phosphatase; AML1, acute myeloid leukemia 1; Ang, angiopoietin; EC, endothelial cell; EPC, endothelial progenitor cell; HC, hematopoietic cell; HPC, hematopoietic progenitor cell; HRP, horseradish peroxidase; HSC, hematopoietic stem cell; MC, mural cell; PB, peripheral blood; PDGF, platelet-derived growth factor; SMA, smooth muscle actin; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor.

Vascular development involves a highly organized sequence of events, such as vasculogenesis and angiogenesis. During blood vessel formation, the first step is the differentiation of endothelial cells (ECs) and mural cells (MCs), such as smooth muscle cells (SMCs) and pericytes, from vascular stem cells expressing vascular endothelial growth factor (VEGF) receptor 2 (Flk-1; reference 1). This is followed by proliferation and migration of ECs and eventual formation of endothelial tubes resulting in formation of the primary vascular plexus. This event is termed vasculogenesis. Subsequently, for the adjustment of tissue-specific oxygen and nutrient supply, the primary vascular plexus is remodeled by sprouting or nonsprouting angiogenesis, fusion of vessels, and regression. Finally, the maturation of nascent vasculature is accomplished by recruitment and adhesion of MCs to ECs. This final step allows generation of an extracellular matrix and specialization of the vessel wall for structural support and regulation of vessel function (2).

It is clear that maintenance of cell adhesion between ECs and MCs is necessary to safeguard against pathogenic malformation as well as aging because it is well known that pericyte loss leads to abnormal blood vessel formation in many diseases, such as diabetic microangiopathy, cancer, venous malformation, and so on (3, 4). Therefore, to address the development of therapy for such diseases, it is important to investigate the development of MCs and manipulate MCs in vitro for potential use in regeneration therapy and research into molecular targets of drugs.

Emerging evidence indicates that platelet-derived growth factor (PDGF)-B plays a critical role in the recruitment of MCs to newly formed vessels (5, 6). VEGF and PDGF-B are likely to participate in regulating both MC and

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EC survival. PDGF-B induces VEGF expression in SMCs and cultured pericytes, and VEGF is known to protect ECs from apoptosis (7, 8). Angiopoietin (Ang)-1, a ligand for Tie2 expressed on ECs, is produced from MCs and promotes selective cell adhesion between MCs and ECs, which is mediated by the activation of integrin on ECs (9, 10). During angiogenesis, prolonged expression of Ang-2 from ECs inhibits MC adhesion to ECs resulting in regression of newly developed blood vessels (11). Genetic ablation of Ang-1 (9) or Tie2 (12, 13) and overexpression of Ang-2 (14), an antagonist for Ang-1, in ECs of mice lead to dissociation between MCs and ECs. These observations strongly suggest a central role for the Tie2–Ang system in the stabilization and destabilization of blood vessels.

Although the molecular mechanism of adhesion between MCs and ECs is gradually becoming better understood, little is known about how MCs facilitate the angiogenic sprout. In the larger vessels, mesenchymal cells surrounding ECs differentiate into MCs, mainly SMCs, which then adhere to ECs. On the other hand, in the case of newly developed capillaries that sprout from preexisting vessels, the common view is that MC recruitment lags behind that of ECs in the angiogenic process. This view is based on studies of retinal angiogenesis (15). However, an opposing view states that in the corpus luteum, MCs are the first vascular cells to migrate into the hypoxic region and might promote migration of ECs (16). Thus, the relationship between ECs and MCs may be different in various angiogenic contexts based on the origin of MCs. There are four possible origins for MCs, namely, mesenchymal cells, ECs, neural crest cells, and adult BM hematopoietic stem cell (HSC) populations (17). However, in the case of pericytes phenotypically overlapping with SMCs, the origin is not defined yet. Pericytes are a source of SMCs as well as fibroblasts and have been reported to show self-renewal activity, suggesting that they may be a stem cell population (18).

We previously reported that HSCs migrate into the avascular area and promote sprouting angiogenesis by producing Ang-1, a chemoattractant for ECs (19). Because Ang-1 production and self-renewal activity are common features of HSCs and pericytes, and moreover, HSCs have a capacity to adhere with ECs (10), it is possible that in the physiologic situation the HSC population differentiates into SMCs through the pericyte lineage.

Recently, it was reported that the HSC population designated as $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ in the BM of adult mice differentiates into SMCs and are incorporated into blood vessels in pathologic situations, such as atherosclerosis, postangioplasty restenosis, and graft vasculopathy (20). However, whether the derivation of MCs from the HSC population is induced in normal physiological conditions or not, the hierarchical differentiation pathway of HSCs into MCs is not yet fully clear. Therefore, in this study, we investigated the role of HSCs in the stability of blood vessels using acute myeloid leukemia 1 (AML1) null embryos, which lack definitive-type HSC development. We studied the molecular and environmental cues necessary for the derivation of MCs from the HSC population. Using mice, we report that HSCs actually change their pathway of differentiation into a MC population through a $CD45^+CD11b^+$ cell lineage in vitro and in vivo, and play an important role for the stabilization of newly developed blood vessels in physiologic and pathologic situations.

RESULTS

HSC population differentiates into MCs during embryogenesis

We previously reported that HSCs appear in the brain ectodermal layer during embryogenesis before the appearance of ECs to form the capillary network at this site (19). These HSCs produce Ang-1, promote sprouting angiogenesis from the peri-ectodermal vascular plexus, and finally locate near newly developed blood vessels. Without HSC development in AML1 mutant embryos, vascular network formation in the ectodermal layer was insufficient and hemorrhage occurred from the disorganized blood vessels at the embryonic day 12.5 (E12.5) stage (21, 22). Based on these observations, we hypothesized that the HSC population might be associated with the stability of blood vessels. Stability or maturation of blood vessels is closely related to MC attachment to ECs. Therefore, we first studied the development of MCs in the ectodermal layer focusing on the localization of HSCs or hematopoietic progenitor cells (HPCs) using wild-type and AML1 mutant embryos.

At E10.5, the $c\text{-}Kit^+$ HSC/HPC population is located in the proximity of ECs in the brain ectodermal layer (Fig. 1 a); however, in this stage, we could not detect the smooth muscle actin $(SMA)^+$ or $desmin^+$ MCs (Fig. 1 b; desmin not depicted). 1 d later at E11.5, we observed that some HSCs/HPCs adhered to ECs (Fig. 1 d) and MCs adhering to ECs also first appeared in this stage (Fig. 1 e). At E12.5, adhesion of HSCs to ECs was still observable (Fig. 1 g) and most of the capillaries contained MCs (Fig. 1 h). On the other hand, in the case of AML1 mutant embryos, the HSC population marked by c-Kit (not depicted) and MCs (Fig. 1, c and f) could not be detected in the ectodermal layer at E10.5 and E11.5.

Based on these observations, it appears that the HSC/HPC population differentiates into MCs and promotes the stability of blood vessels. To confirm this observation, we attempted to clarify whether a purified HSC population in vitro could differentiate into MCs or not. As showed in Fig. 1 i, we sorted the HSC/HPC population designated as Lin^- $c\text{-}Kit^+$ among $CD45^+$ hematopoietic cells (HCs) from the ectodermal layer of E12.5 embryos and cultured them on fibronectin-coated culture plates in the presence of PDGF-BB. From 10^4 HSCs, only a small number of cells ($0.12 \pm 0.02\%$, $n = 3$) were found to be able to adhere to the culture plate and produce colonies; however, almost all colonies contained $PDGFR\beta^+$ (Fig. 1 j) and SMA^+ (Fig. 1, k and l) cells. As previously reported in the adult (23), we found that ECs expressing CD31/PECAM-1 or Flk-1 also developed from

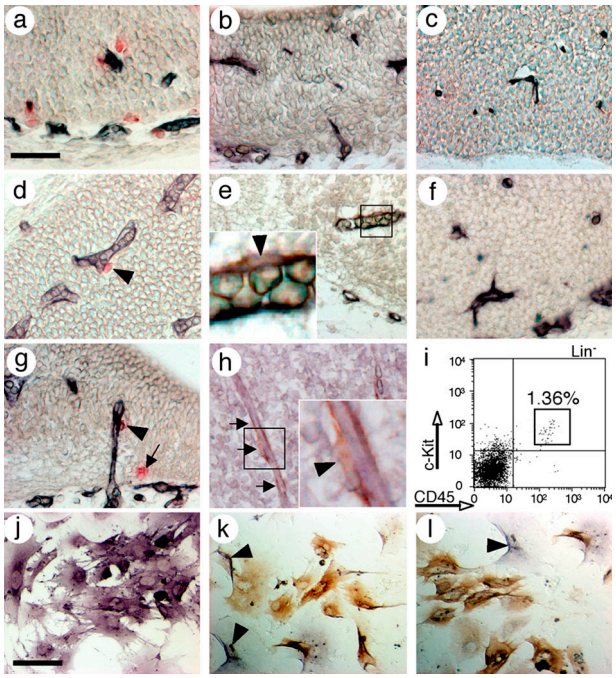

Figure 1. HSC population in the embryonic brain can give rise to MCs. (a–h) Brain sections at E10.5 (a–c), E11.5 (d–f), and E12.5 (g and h) from wild-type mice (a, b, d, e, g, and h), and AML1 mutant mice (c and f) were dual stained with anti–c-Kit mAb (red) and anti-CD31 mAb (dark blue; a, d, and g) or with anti-SMA mAb (brown) and anti-CD31 (dark blue; b, c, e, f, and h). Inset (e and h) shows high power view indicated by box. Bar in panel a, 50 μm. (i) FACS analysis of HSC/HPC population existing in the mouse brain at E12.5. The number in the top-right quadrant indicates the percentage of c-Kit$^+$ CD45$^+$ cells among Lin$^-$ cells. The data is representative of three independent experiments. (j–l) Phenotype of cultured HSC/HPC populations in vitro. The HSC/HPC population indicated by the box in panel i was cultured on fibronectin-coated culture plates for 14 d and stained with anti-CD31 (dark blue; j), anti-SMA mAb (brown; k and l), anti–PECAM-1 mAb (dark blue; k), or anti–Flk-1 mAb (dark blue; l). Arrows (k and l) indicate cells expressing CD31 or Flk-1, respectively. Bar in panel j, 30 μm.

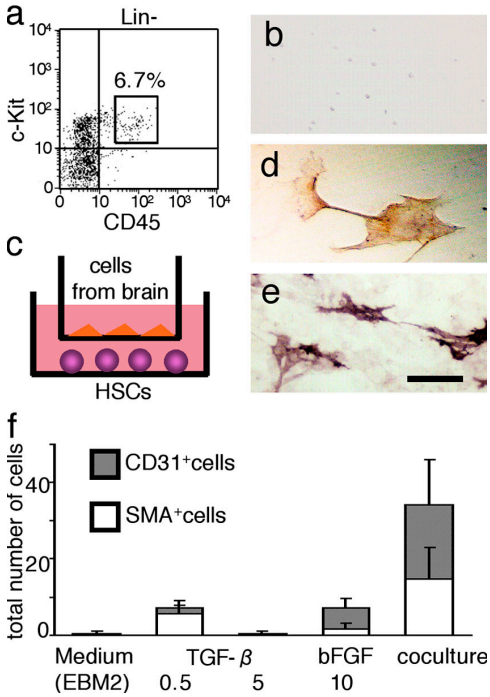

Figure 2. Induction of differentiation of the HSC/HPC population existing in the fetal liver into MCs. (a) FACS analysis of the HSC/HPC population existing in the mouse fetal liver at E12.5. The number in the top-right quadrant indicates the percentage of c-Kit$^+$ CD45$^+$ cells among Lin$^-$ cells. The data is representative of three independent experiments. (b–f) The HSC/HPC population indicated by the box in panel a was cultured with or without cells from the brain in the presence of PDGF-BB and VEGF. (b) HSCs/HPCs alone cultured for 14 d were stained with anti-SMA mAb. (c) Schematic presentation of the coculture system with HSCs/HPCs from the fetal liver and cells from the brains of E12.5 mouse embryos. (d and e) HSCs/HPCs cultured with cells from the brain were stained with anti-SMA mAb (brown; d) or anti–CD31 mAb (dark blue; e). Bar in panel e, 30 μm. (f) Growth factors affecting the induction of HSCs/HPCs into MCs or ECs. HSCs/HPCs indicated by the box in panel a were cultured in the basic medium alone (EBM2), in EBM2 with 0.5 or 5 ng/ml TGF-β or 10 ng/ml bFGF, or with cells from the brain as described above. Cells adhering to culture plates were stained with anti–CD31 mAb or anti-SMA mAb, and the number of positively stained cells were determined. Results are expressed as the mean ± SD (n = 5).

the HSC population in the embryo in the presence of PDGF-BB and VEGF (Fig. 1, k and l).

Environmental molecular cues are required for the differentiation of MCs from the HSC population

During early embryogenesis, HSCs develop in the AGM region surrounded by the aorta, gonads, mesonephros, dorsal aorta, and the omphalomesenteric artery, migrate to the fetal liver for their expansion, and then widely spread into the whole body. As has been reported, the HSC population has a plasticity to differentiate into cells other than HCs (24–26). However, whether such plasticity occurs during embryogenesis is not clear. If such transdifferentiation is observed during embryogenesis, differentiation of MCs from HSCs seems to be a natural course of the programmed differentiation pathway for organogenesis. To find out whether HSCs located in the regions other than the brain can also differentiate into MCs, the HSC population was sorted from E12.5 fetal liver as a major hematopoietic site, fractionated using identical markers (Lin$^-$c-Kit$^+$CD45$^+$; Fig. 2 a), and cultured in the same culture condition as described above. Although a small number of HSCs adhered to the culture plate, we could not detect SMA$^+$ (Fig. 2 b) nor desmin$^+$ (not depicted) MCs. However, when HSCs/HPCs from fetal liver were cocultured with cells from the brain ectodermal layer of the E12.5 embryo, they differentiated into MCs (Fig. 2 d) as well as ECs (Fig. 2 e). These findings suggested that the environmental molecular cues produced from cells composing the ectodermal layer must regulate the differentiation of HSCs/HPCs into MCs. Among several growth factors examined, we found that TGF-β or bFGF has a

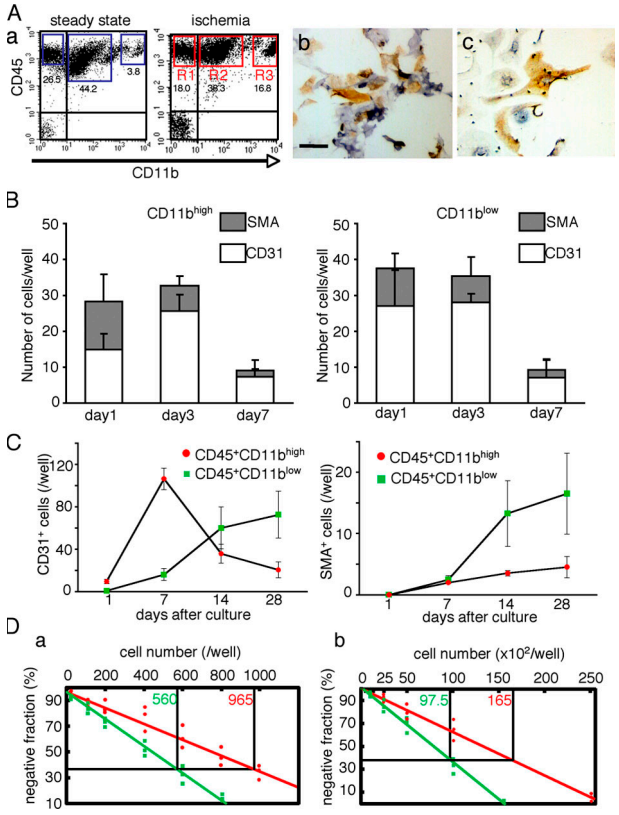

Figure 3. CD45$^+$CD11b$^+$ cells existing in the PB under the hypoxia differentiate into MCs. (A) FACS analysis of HCs in the PB of mice. Mononuclear cells dual stained with anti-CD45 mAb and anti-CD11b mAb under normoxic (steady-state) conditions and ischemia. The numbers indicate the fraction of the cells in each box in the total mononuclear cells in the PB. Cells fractionated as CD45$^+$CD11b^{low} (b) and CD45$^+$CD11b^{high} (c) were sorted separately and cultured in the presence of VEGF and PDGF-BB. Cultured cells on fibronectin-coated plates were stained with anti-SMA mAb (brown) and anti-Flk-1 mAb (dark blue). Bar, 25 μm. (B) Development of CD31$^+$ ECs and SMA$^+$ SMCs from CD11b^{high} and CD11b^{low} cells in the PB of mice in which ischemia had been induced by ligation of the femoral artery. CD11$^+$ cells were collected from the PB on days 1,3, and 7 after ischemia was induced and cultured for 14 d. (C) Time course of EC (left) and MC (right) development from CD11b^{low} cells (green square) or CD11b^{high} cells (red circle). Results are expressed as the mean ± SD ($n = 5$). (D) Limiting dilution analysis of CD11b^{low} cells (green square) or CD11b^{high} cells (red circle) for differentiation into ECs (a) and MCs (b). The frequency of cell differentiation into ECs or MCs is depicted on the graph based on the Poisson analysis.

weak capacity to promote the differentiation of fetal liver HSCs into MCs (Fig. 2 f).

Differences between CD45$^+$CD11b^{low} cells and CD45$^+$CD11b^{high} cells for development into MCs

In the steady-state in the normal animal, mononuclear cells existing in peripheral blood (PB) barely differentiate into MCs or ECs if isolated and cultured under the same culture conditions as was used for HSCs/HPCs from the brain of embryos (not depicted). Therefore, appropriate molecular signals may change the fate of HSCs into MCs in the adult as well as in the embryo. To address this issue, we induced hypoxia in the hind limb of mice by ligation of the femoral artery and observed whether the HCs in the PB can differentiate into MCs. The monocyte/macrophage lineage has been suggested to differentiate into ECs (27, 28) and, moreover, MCs and ECs are thought to develop from common progenitors (1); therefore, at first, we used CD45 and CD11b as markers of vascular progenitor cells.

FACS analysis showed that there were three populations among CD45$^+$ cells, namely, CD11b$^-$, CD11b^{low}, and CD11b^{high} in hypoxia-induced PB (Fig. 3 A, a). The CD45$^+$CD11b^{low} (CD11b^{low}) cells expressed Sca-1 strongly and c-Kit weakly; however, CD45$^+$CD11b^{high} (CD11b^{high}) cells expressed both of them at a low level (Table I). Moreover, analysis of the CFUs in culture (CFU-c) revealed that CD11b^{low} cells have a higher capability to form CFU-GEMM, BFU-E, and CFU-GM compared with CD11b^{high} cells (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1). These observations suggested that phenotypically, the CD11b^{low} population belongs to the HSC/HPC population and the CD11b^{high} population belongs to the mature monocyte/macrophage population, and they highly expressed CD11c and CD14 (Table I) as previously reported (29, 30).

Next, we cultured CD11b^{low}, CD11b^{high}, and CD45$^+$ CD11b$^-$ (CD11b$^-$) cells from hypoxia-induced PB in the same culture condition as described above. Both CD11b^{low} and CD11b^{high} cells produced SMA$^+$ MC lineage cells (Fig. 3 A, b and c). Although the EC lineage derived from both CD11b^{high} and CD11b^{low} populations expressed CD31 strongly (not depicted), the EC lineage from the CD11b^{high} population expressed Flk-1 very weakly compared with that from the CD11b^{low} population (Fig. 3 A, c). We confirmed that MCs or ECs derived from CD11b$^+$ cells expressed other vascular cell–specific markers, PDGFRβ and desmin or VE-cadherin and vWF, respectively (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1). On the other hand, CD11b$^-$ cells differentiated into neither Flk-1$^+$/CD31$^+$ cells nor SMA$^+$ cells (not depicted). We then examined the time of appearance of vascular progenitor cells in CD11b^{low} and CD11b^{high} populations abundantly after ischemia was induced. For this purpose, sorted 10^5 CD11b^{low} and CD11b^{high} populations of PB on days 1, 3, and 7 after ischemia was induced were cultured on a fibronectin-coated dish. After 14 d of culturing, we evaluated EC and SMC development from two populations. We detected CD31$^+$

Table I. Expression of monocyte makers in CD11b subpopulation

	CD11b$^-$ (%)	CD11b^{low} (%)	CD11b^{high} (%)
c-Kit	0.5 ± 0.1	2.0 ± 0.3	0.7 ± 0.3
Sca-1	50.6 ± 2.8	94.3 ± 6.5	13.7 ± 1.6
CD11a	84.6 ± 6.8	83.7 ± 10.5	95.0 ± 9.7
CD11c	0.4 ± 0.1	3.2 ± 1.3	71.5 ± 8.6
CD14	3.3 ± 1.1	7.0 ± 1.8	77.4 ± 9.2
CD18	5.5 ± 1.8	25.2 ± 5.6	95.1 ± 10.1

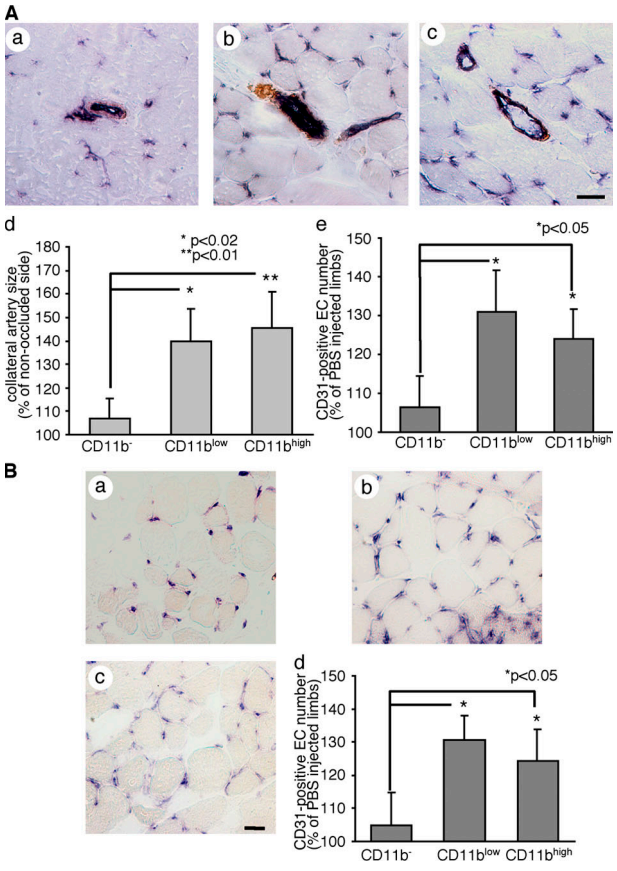

Figure 4. Contribution of CD45$^+$CD11b$^+$ cells for neovascularization. CD45$^+$CD11b$^-$ or CD45$^+$CD11b$^+$ cells were sorted as described in Fig. 3 A from the PB of mice in which ischemia had been induced by ligation of the femoral artery. Cells thus obtained were injected into the ischemic thigh muscles of other mice that had their femoral arteries ligated and resected. (A) Evaluation of the size of collateral artery and the vessel number in the adductor muscle. Sections of adductor muscle injected with CD45$^+$CD11b$^-$ (a), CD11b^{low} (b), or CD11b^{high} (c) cells were stained with anti-CD31 antibody. Bar in panel c, 50 μm. (d) Size of collateral artery counted in five random fields. (e) Number of capillaries counted in five random fields. (B) Evaluation of the number of capillaries in the gastrocnemius muscles. Sections of adductor muscle injected with CD45$^+$CD11b$^-$ (a), CD11b^{low} (b), or CD11b^{high} (c) cells were stained with anti-CD31 antibody. Bar in panel c, 50 μm. (d) Number of capillaries counted in five random fields.

EC and SMA$^+$ SMC development abundantly in the cultures of both CD11b^{high} and CD11b^{low} populations from PB 1 and 3 d after ischemia; however, the number of ECs and SMCs developed from CD11b^{high} and CD11b^{low} populations on day 7 after ischemia decreased (Fig. 3 B). This indicated that mobilization of vascular progenitor cells designated as CD45$^+$CD11b^{low} or CD45$^+$CD11b^{high} in PB was induced in the early stage of ischemia. Therefore, in the following experiments, we used CD45$^+$CD11b$^+$ (CD11b$^+$) cells from PB on day 3 after ischemia was induced.

To determine the time course of development and growth of the MC and EC lineages, we cultured CD11b^{high} and CD11b^{low} populations for 28 d (Fig. 3 C). CD11b^{high} cells adhering to culture plate showed a spindle-like shape within 24 h and produced a large number of CD31$^+$ cells at day 7; however, the number of CD31$^+$ cells decreased gradually and this was caused by apoptosis of the ECs (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1). In contrast, CD11b^{low} cells formed a spindle-like shape around day 7 and the number of CD31$^+$ cells gradually increased (Fig. 3 C). Interestingly, the growth kinetics of MC development is much higher for CD11b^{low} cells than CD11b^{high} cells (Fig. 3 C).

Next, we estimated the incidence of EC or MC development from CD11b^{low} or CD11b^{high} cells by limiting dilution analysis and found that 1 out of 560 CD11b^{low} cells and 1 out of 965 CD11b^{high} cells can differentiate into ECs, whereas, 1 out of 9,750 CD11b^{low} cells and 1 out of 16,500 CD11b^{high} cells differentiate into MCs. The low incidence of MC or EC development from CD11b^{low} and CD11b^{high} cells is likely due to the limitation of the in vitro culture system. These findings suggested that CD11b^{low} cells are more immature than CD11b^{high} cells and have a higher potential to differentiate into MCs. Additionally, in sham-operated mice, i.e., under normoxic conditions, the FACS staining profile of CD45 and CD11b markers in PB was similar to that under ischemic conditions (Fig. 3 A, a). However, we determined that 1 among 4,380 or 1 among 68,200 CD11b^{low} cells differentiated into ECs or MCs, respectively, and that 1 among 5,250 or 1 among 89,100 CD11b^{high} cells differentiated into MCs or ECs, respectively. Collectively, these results suggested that cells from the CD11b$^+$ population are capable of differentiating into MCs in adulthood and such differentiation capacity is induced by some molecular cues that exist in pathologic conditions such as ischemia.

The contribution of CD45$^+$CD11b$^+$ cells in ischemic limbs

We studied the mechanism whereby CD11b$^+$ cells contributed to the improvement of ischemia in the hind limbs. To achieve this, CD11b$^-$, CD11b^{low}, and CD11b^{high} cells were isolated from mice that had ischemia induced in the hind limbs and were injected into the thigh muscles of the hind limbs of other mice in which ischemia had been induced by occlusion of the femoral arteries. 14 d after injection of the cells, we dissected the adductor and gastrocnemius muscle and evaluated the collateral artery size and capillary number. The collateral artery size and the CD31$^+$ capillary number in the adductor muscle increased after injection of CD11b^{low} or CD11b^{high} cells (Fig. 4 A, b and c) compared with those of mice injected with CD11b$^-$ cells (Fig. 4 A, a, d, and e). Moreover, improvement of blood flow in the upper limbs by injection of CD11b^{low} or CD11b^{high} cells enhanced capillary formation in the gastrocnemius muscles and inhibited the atrophy that was observed in the gastrocnemius muscle injected with CD11b$^-$ cells (Fig. 4 B). These results indicated that CD11b^{low} and CD11b^{high} cells equally enhanced capillary formation and improved ischemic damage.

The mechanism of recruitment of CD11b population into ischemic limbs

To determine whether intravenous injection of $CD11b^+$ cells can lead to selective homing of these cells to the ischemic limb, we injected from the tail vein $CD11b^-$, $CD11b^{low}$, or $CD11b^{high}$ cells derived from ischemia-induced green mice (31) that express GFP ubiquitously into ischemia-induced wild-type mice and then observed the localization of the injected CD11b subpopulation in the adductor muscles after 3 d. The results showed that $CD11b^-$ cells did not appear in the ischemic adductor muscle (Fig. 5 A, a); however, $CD11b^{low}$ and $CD11b^{high}$ cells were randomly distributed around the vessels in the ischemic limb (Fig. 5 A, b and c). $CD11b^{high}$ cells tended to be more abundant compared with $CD11b^{low}$ cells, but this difference was not statistically significant (Fig. 5 A, d). Moreover, we confirmed that endogenous $CD11b^+$ cells were recruited into and adhered to the vessel wall in the ischemic limbs (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1).

Next, we examined the mechanisms that support the recruitment of CD11b cells to ischemic limbs. We extracted mRNA from the nonischemic and ischemic muscle to identify the critical chemokines, and we used $CD11b^-$, $CD11b^{low}$, and $CD11b^{high}$ cells in the PB of ischemia-induced mice to identify the chemokine receptors for the recruitment of these cells to the ischemic site. Among the various chemokines, CCL2 and SDF-1α are major chemokines in the monocyte and HSC lineages (32), and VEGF-A is also known as one of the most potent chemoattractant proteins in those fractions (23). Therefore, we investigated the role of these factors and their receptors. The results indicated that CCL2, SDF-1α, and VEGF-A were up-regulated in ischemic muscles and their receptors; namely, CCR-2, CXCR4, and VEGFR-1 were detected on $CD11b^{low}$ and $CD11b^{high}$ fractions but not on $CD11b^-$ fractions (Fig. 5 B).

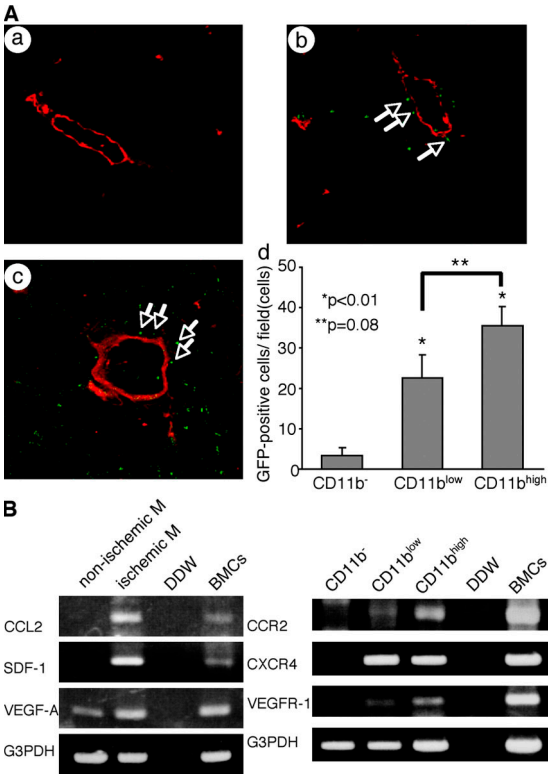

Figure 5. Recruitment of the CD11b subpopulation into ischemic lesions. (A) Sections of adductor muscle from mice in which ischemia had been induced by ligation of the femoral artery and that were injected intravenously with $CD45^+CD11b^-GFP^+$ (a), $CD11b^{low}GFP^+$ (b), or $CD11b^{high}GFP^+$ (c) cells were studied. Arrows indicate GFP^+ cells. (d) Number of GFP^+ cells counted in five random fields. (B) RT-PCR analysis of CCL2, SDF-1, and VEGF-A expression in ischemic muscle (left, ischemic M) and nonischemic muscle (non-ischemic M) or of CCR2, CXCR4, and VEGFR-1 expression in $CD11b^-$, $CD11b^{low}$, and $CD11b^{high}$ cells (right). BM cells (BMCs) were used as positive control.

In vivo incorporation of $CD45^+CD11b^+$ cells into vascular cells

Next, we investigated the role of $CD11b^+$ cells in the process of neovascularization in vivo. To achieve this, $CD11b^+$ or $CD11b^-$ cells isolated from green mice that had ischemia induced in the hind legs were injected into the thigh muscle of the hind limbs of mice in which ischemia had been induced by occlusion of the femoral artery. 3 d after injection of cells, GFP^+ cells were seen to be randomly distributed in the ischemic muscle. However, after 14 d of transplantation, most GFP^+ cells remaining in the ischemic muscle were associated with blood vessels. As observed in vitro, $CD11b^+$ cells had the potential to differentiate into ECs as indicated by the incorporation of GFP^+ cells into newly developed blood vessels as ECs (Fig. 6 A, a–c).Moreover, GFP^+ cells were seen to adhere to ECs and expressed PDGFRβ (Fig. 6 B, a–c) and SMA (not depicted) in the ischemic tissues, suggesting that $CD11b^+$ cells could differentiate into MCs (Fig. 6 B, a–c). As observed in the in vitro experiments, $CD11b^-$ cells did not contribute to ECs or MCs in the ischemic muscle (Fig. 6 A, d–f, and B, d–f).

Additionally, we compared the long-term contribution of transplanted $CD11b^{high}$ or $CD11b^{low}$ cells for vascular cells. The number of $CD11b^{high}$ cells that formed ECs and MCs after 6 mo of transplantation decreased significantly compared with that after 2 wk of transplantation. On the other hand, although the number of ECs or MCs decreased, $CD11b^{low}$ cells were capable of forming vascular cells for a longer period than $CD11b^{high}$ cells (Fig. 6 C).

To assess functional recovery, we observed the behavior of the mice. In the first 2 wk, movement of ischemic legs in mice injected with $CD11b^{high}$ cells declined slightly compared with that of mice injected with $CD11b^{low}$ cells. However, such impairment was gradually reduced and movement of legs became comparable between $CD11b^{high}$-injected mice and $CD11b^{low}$-injected mice after 1 mo. Although the density of blood vessels in the ischemic muscles of $CD11b^{high}$ cell–injected mice was nearly 15% lower relative to that of mice injected with $CD11b^{low}$ cells after 6 mo of transplantation

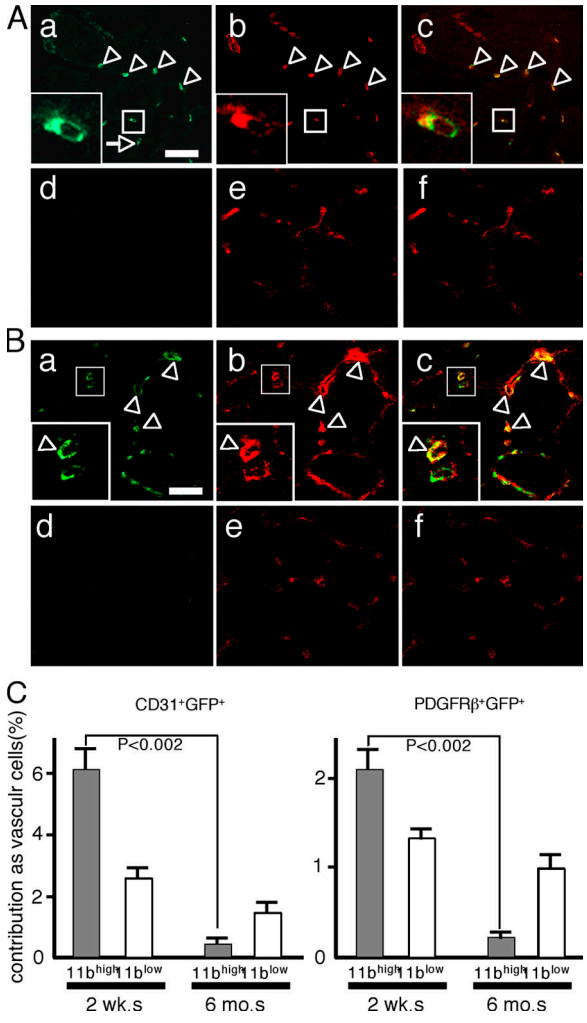

Figure 6. Development of CD45$^+$CD11b$^+$ cells into ECs and MCs in newly developed blood vessels of ischemic thigh muscles. Differentiation into ECs (A) or MCs (B) of CD45$^+$CD11b$^-$ or CD45$^+$CD11b$^+$ cells injected into ischemic thigh muscles as described in Fig. 4 was observed using laser-scanning confocal microscopy. (A) Section from a thigh muscle injected with CD45$^+$CD11b$^+$ (a–c) or CD45$^+$CD11b$^-$ (d–f) cells from green mice. (a and d) GFP (green), (b and e) anti-CD31 mAb (red), (c) merged images of panels a and b, (f) merged image of panels d and e. Arrowheads indicate GFP$^+$CD31$^+$ ECs. Insets show high power view of area indicated by box. Bar, 50 μm. (B) Section from a thigh muscle injected with CD45$^+$CD11b$^+$ (a–c) or CD45$^+$CD11b$^-$ (d–f) cells from green mice. (a and d) GFP (green), (b and e) anti-PDGFRβ mAb (red), (c) merged image of panels a and b, (f) merged image of panels d and e. Arrowheads indicate GFP$^+$PDGFRβ$^+$ MCs. Insets show high power view of area indicated by box. Bar, 50 μm. (C) Development of CD11b^{high} or CD11b^{low} cells into vascular cell in the ischemic thigh muscle at 2 wk (wk.s) or 6 mo (mo.s) after injection of cells. Percentage of vascular cells originating from CD11b^{high} or CD11b^{low} cells was expressed as percentage of total capillaries counted in 30 random fields. In brief, when the GFP signal was detected in a cross section of a capillary as seen in the insets of A (a–c), this capillary was counted as being formed by the injected cells.

(not depicted), we concluded that the difference in the potential of CD11b^{high} and CD11b^{low} cells to restore the function of ischemic muscle was not significantly different.

CD45$^+$CD11b^{low} cells can promote nonleaky blood vessel formation through Ang-1

Next, we examined how CD11b^{low} and CD11b^{high} cells affected the vascular phenotype. CD11b^{low} cells express c-Kit and Sca-1 and are thought to belong to the HSC/HPC population. This is in contrast to CD11b^{high} cells, which are the so-called mature macrophages or monocytes. Interestingly, we found that postoperatively, in the mice injected with CD11b^{high} cells, the open incision persisted for 14 d with effusion of infiltrate due to the trauma. On the other hand, such infiltration was not observed in mice injected with CD11b^{low} cells, and the incision closed within 14 d (not depicted).

Based on these observations, we hypothesized that CD11b^{high} or CD11b^{low} cells produce molecules that regulate permeability. It was previously reported that VEGF-mediated hyperpermeability was inhibited by Ang-1 (33). Furthermore, we reported that the HSC population produces Ang-1 strongly and lack of HSCs in AML1-deficient mice resulted in the rupture of blood vessels during embryogenesis (19). The phenotype of CD11b^{low} cells is similar to the HSC/HPC population; therefore, we speculated that CD11b^{low} cells contribute to the maturation of blood vessels, such as nonleaky blood vessels. Thus, we compared the expression of VEGF and Ang-1 in CD11b^{high} cells and CD11b^{low} cells. As expected, the CD11b^{low} cells as well as the HSC population had high levels of expression of Ang-1, but CD11b^{high} cells did not (Fig. 7 a). Indeed, the expression level of Ang-1 in CD11b^{low} cells was ~10 times higher than in CD11b^{high} cells by quantitative RT-PCR analysis (not depicted). Conversely, higher expression of VEGF was detected in CD11b^{high} cells compared with CD11b^{low} cells.

We then investigated the effect of injection of CD11b^{high} or CD11b^{low} cells on the dermis of the animals. There was edema in the dermis of the hypoxia-induced mice injected with CD11b^{high} cells (Fig. 7 b). In contrast, such edema was not observed in mice injected with CD11b^{low} cells (Fig. 7, c and e). As expected, injection of CD11b^{low} cells from Ang-1 mutant mice (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1; reference 9) could not prevent edema (Fig. 7, d and e), although the CD11b^{low} cells could incorporate into the wall of blood vessels (Fig. S4). Additionally, the edema observed in limbs injected with CD11b^{high} cells was suppressed by simultaneous injection with CD11b^{low} cells (not depicted).

Next, we confirmed that the leakiness of blood vessels induced by tail vein injection of lectin into mice increased in the mice injected with CD11b^{high} cells (Fig. 7 f), but not in mice that received CD11b^{low} cells (Fig. 7 g) in the hind limb ischemia model. Such a beneficial effect of CD11b^{low} cells from wild-type animals was not observed by the injection of CD11b^{low} cells from Ang-1 mutants (Fig. 7 h). Although CD11b^{low} cells and CD11b^{high} cells can contribute

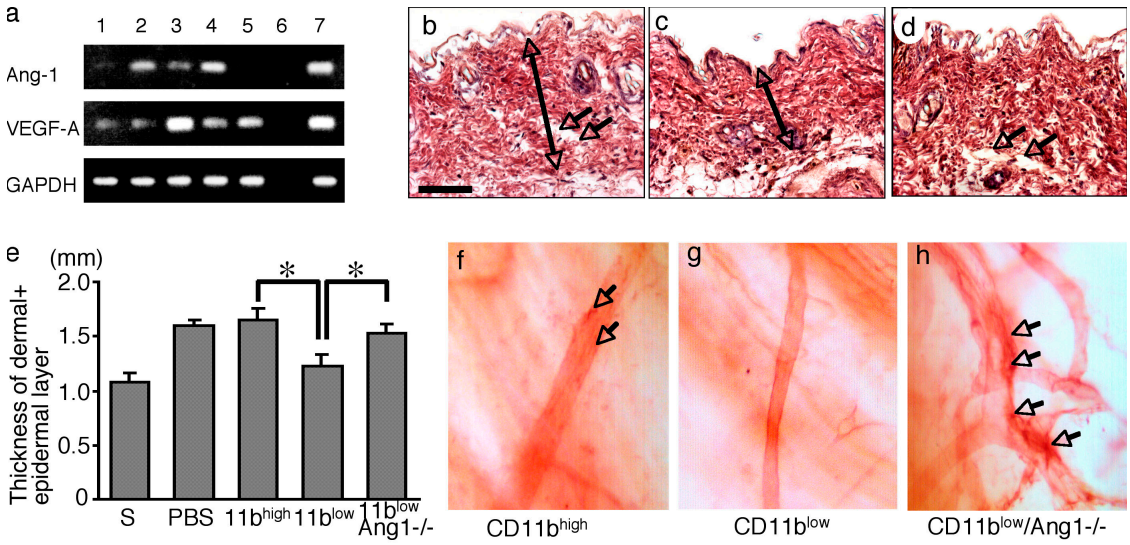

Figure 7. Comparison of permeability of blood vessels in animals injected with CD11b^{low} and CD11b^{high} cells. (a) RT-PCR analysis of Ang-1 or VEGF-A expression in several hematopoietic lineages. Lane 1, CD45$^+$Lin$^+$ cells; lane 2, CD45$^+$Lin$^-$ cells; lane 3, CD45$^+$CD11b^{high} cells; lane 4, CD45$^+$CD11b^{low} cells; lane 5, CD45$^+$CD11b^{low} cells from Ang-1 mutants; lane 6, distilled water; lane 7, Lin$^-$c-Kit$^+$Sca-1$^+$ cells in BM. (b–h) Mice in which ischemia was induced by the occlusion of the femoral artery were treated with CD11b^{low} or CD11b^{high} cells sorted as described in Fig. 3 A or Fig. S4. (b–d) Hematoxylin and eosin staining of skin near the limb 7 d after injection with CD11b^{high} (b), CD11b^{low} (c), and CD11b^{low} cells from Ang-1 mutants (d). Arrows in panels b and d indicate edematous space. Bar, 500 μm. (e) Thickness of the dermal and epidermal layers after treatment with CD11b^{high} or CD11b^{low} cells. Thickness of the dermal and epidermal layers of the limbs was measured at a distance of 1 cm from the knee and compared with that in mice treated with PBS or with sham (S)-operated mice. Results are expressed as the mean ± SD ($n = 5$). *, $P < 0.02$. (f–h) Leakage site in thigh muscle microvessels stained by perfusion of lectin (ricin) after injection of CD11b^{high} (f), CD11b^{low} (g), and CD11b^{low} cells from Ang-1 mutants (h). Arrows indicate the exposed basement membrane, which shows vascular leaks.

to vascular cells similarly, these data suggest that blood vessel maturation might be more effectively induced by CD11b^{low} cells by the ability to differentiate into MCs, long-term survival after differentiation into MCs, and inhibition of leakiness through Ang-1.

DISCUSSION

In this study, we found that HSCs or CD11b^{low} cells played critical roles in promoting stable blood vessel formation by their differentiation into MCs and by the production of Ang-1 in both physiological and pathological conditions. Although it has been reported that ECs are developed from HSC populations through the monocyte lineage (27, 28), we found that MCs are also differentiated from the hematopoietic lineage through the same pathway as ECs by using CD45 and CD11b, well-known specific markers expressed on HCs and the monocyte/macrophage lineage, respectively. In particular, we showed differences between CD11b^{low} cells and CD11b^{high} cells in blood vessel formation. One such difference was that CD11^{low} cells have a long-term regeneration capacity for neovascularization by differentiating into ECs and MCs, but CD11b^{high} cells do not. The other is that CD11b^{high} cells induced the formation of leaky blood vessels, but CD11b^{low} cells did not. Thus, CD11b^{low} cells might promote the stability of newly developed blood vessels.

Progenitor cells for ECs or MCs have been suggested to exist in adult BM, and several reports have suggested that these cells develop into endothelial progenitor cells (EPCs) in the neovascularization of tumors and ischemic regions (34, 35). Moreover, the HSC population in the adult BM was reported to incorporate into blood vessels as MCs in atherosclerotic plaques and in transplanted hearts. Sata et al. (20) suggested that vascular SMC progenitors also originated from the Lin$^-$c-Kit$^+$Sca-1$^+$ HSC population. At the moment, it is not clear whether single Lin$^-$c-Kit$^+$Sca-1$^+$ cells can give rise to the three different cell types, namely, HCs, ECs, and MCs. However, in this study, we showed that the latter two populations develop from the HC lineage by using CD45 as a marker expressed on HCs but not on ECs nor MCs.

Although CD11b$^+$ cells could give rise to MCs as well as ECs, there were differences between CD11b^{high} and CD11b^{low} cells. CD11b^{high} cells rapidly differentiated into CD31$^+$ EC-like cells in vitro and were found to incorporate into newly developed blood vessels as ECs in an in vivo ischemia model. However, they showed low frequency for differentiation into MCs and ECs, and MCs from CD11b^{high} cells could not survive for the long term, both in vitro and in vivo. In contrast, although CD11b^{low} cells took more time for their differentiation into ECs, they differentiated into MCs with high frequency. Both MCs and ECs from CD11b^{low} cells could form vascular cells for the long term.

In the course of angiogenesis, vessel regression is part of the maturation process. Indeed, the number of newly developed blood vessels induced by CD11b$^+$ cells gradually

decreased between 2 wk and 6 mo. In the case of $CD11b^{high}$ cell injection, capillary density after 6 mo was ~70% of that present after 2 wk. On the other hand, in the case of $CD11^{low}$ cells, the capillary density was ~80% at 6 mo compared with that present at 2 wk. It is not clear whether this higher loss of capillary density associated with $CD11b^{high}$ cells reflects higher apoptosis in vivo of ECs generated by $CD11b^{high}$ cells or not; however, this is possible because ECs generated by $CD11^{high}$ cells declined because of apoptosis after 2 wk in vitro (Fig. S2).

Recently, two groups independently reported that there were two populations of cells with the capacity to differentiate into ECs from mononuclear cells in the PB (36, 37). They reported that $CD14^+$ cells or spindle-shape cells in the PB differentiate into ECs rapidly, and such ECs could not survive for the long term. They termed these ECs as early EPCs. On the other hand, ECs derived from $CD14^-$ cells or cells showing a cobblestone appearance grow exponentially, survive for a longer time, and are called late outgrowth ECs or late EPCs. Although they did not observe the differentiation of MCs from EPCs, we assume that the former corresponds to $CD11b^{high}$ cells and the latter to $CD11b^{low}$ cells. Based on a previous report showing that cycling HSCs express CD11b weakly (30), $CD11b^{low}$ cells might belong to the HSC population. However, the results of limiting dilution analysis showing low frequency of differentiation of the HSC population into ECs or MCs suggest that only a part of the immature HSC population expressing CD11b, c-Kit, and Sca-1 can develop into vascular cells.

Additionally, we found that $CD11b^{high}$ cells induced hyperpermeability in this ischemia model, but $CD11b^{low}$ cells did not. In terms of leakiness of blood vessels, Ang-1 produced from MCs has been suggested to induce structurally stable blood vessels by promoting adhesion between MCs and ECs (9). Relative to this, it has been reported that VEGF-mediated hyperpermeability can be suppressed by Ang-1 (33). As far as we observed, $CD11b^{low}$ cells, but not $CD11b^{high}$ cells, express Ang-1 abundantly, but both $CD11b^{low}$ and $CD11b^{high}$ cells express VEGF. We previously reported that the HSC population produces Ang-1 strongly and promotes the remodeling of blood vessels during embryogenesis (19). Moreover, we recently reported that the $CD45^+$c-Kit^+ HSC population regulates caliber change and remodeling of blood vessels located in the fibrous cap of a murine tumor model (38). Collectively, the data strongly suggest that the $CD11b^{low}$ population contains higher amounts of HSCs and promotes remodeling and stability of newly developed blood vessels along with their differentiated MCs as well as the maintenance of Ang-1 production. Recently, there has been interest in the regeneration of blood vessels in ischemic patients using the transplantation of BM cells (39). Our analysis shows that selective injection of BM HSC populations may be effective in achieving blood vessel regeneration.

Although several studies have shown the plasticity of HSCs, the problem of autonomous cell fusion between HSCs and tissue-specific cells has been considered (40, 41). When the HSC population differentiates into another lineage except hematopoietic lineage during embryogenesis or other physiological situations, we consider those events as the natural course of the differentiation pathway in the HSC population. Here we showed that the HSC population in the brain during embryogenesis easily differentiates into MCs as well as ECs. Moreover, coculturing of HSCs from the fetal liver with cells from the brains of embryos is necessary for the differentiation of the HSC population from the fetal liver into vascular cells. This is the first report showing that the HSC population can change their pathway of differentiation to something other than the hematopoietic lineage by receiving adequate molecular cues depending on the tissue demands, such as development and ischemia. Therefore, we propose that differentiation of vascular cells from the HSC population runs a physiologically natural course rather than being attributed to plasticity of the cells. At this moment, the molecular mechanism whereby the HSC population differentiates into MCs as well as ECs is not fully understood. Among various cytokines, we found that TGF-β has a weak capacity for changing the fate of the HSC population into MCs. In the present therapeutic approach to angiogenesis, larger and more structurally stable blood vessel formation, the so-called arteriogenesis, is thought to be important as a new strategy. To address this issue, MC management is essential. In this study, we could generate MC progenitors from the BM HSC population (Figs. S5 and S6, available at http://www.jem.org/cgi/content/full/jem.20050373/DC1), although efficiency was low. Therefore, the use of molecules that are effective in promoting MC development from HSCs may enable the realization of the arteriogenesis goal.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Japan SCL. AML1 mutant mice (22) were provided by T. Watanabe (Tohoku University, Sendai, Japan), green mice that express GFP ubiquitously (23) were provided by M. Okabe (Osaka University, Osaka, Japan), and Ang-1 mutant mice (33) were provided by G.D. Yancopoulos (Regeneron Pharmaceuticals Inc). Animals were housed in environmentally controlled rooms of the animal experimentation facility at Kanazawa University under the guidelines of the Kanazawa University Committee for Animal and Recombinant DNA Experiments.

Immunohistochemistry. Immunohistochemical analyses on tissue sections were performed as described previously (19). The tissue fixation procedures were the same as those described previously (42). The fixed specimens were embedded in OCT compound and sectioned at 7 μm. The following antibody preparations and conjugates were used: anti-CD31 antibody, CD31-biotin (BD Biosciences), anti–c-Kit–biotin (BD Biosciences), anti–VEGFR-2–biotin (BD Biosciences), anti-CD140b antibody (anti-PDGF receptor β; BD Biosciences), and horseradish peroxidase (HRP)-conjugated anti-SMA antibody (Sigma-Aldrich).

In brief, biotinylated anti-CD31, anti–VEGFR-2, and anti–c-kit antibodies were detected by reaction with alkaline phosphatase (ALP)-conjugated streptavidin ABC complex (DakoCytomation), and anti-CD31 and anti-PDGF receptor β antibodies were detected with HRP-conjugated goat anti–rat Igs (Biosource). Diaminobenzidine (DAB; Dojin Chemicals) was used for the HRP color reaction. Fuchsin (New Fuchsin substrate system; DakoCytomation) or 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT; DakoCytomation) was used for the ALP color reaction. For the staining of culture dishes, anti–CD31-biotin,

anti–VEGFR-2–biotin, HRP-conjugated anti-SMA, anti–CD31-PE (BD Biosciences), anti–SMA-Cy3 (Sigma-Aldrich), and anti-GFP (Medical Biological Laboratories Co., Inc.) antibodies were used. Anti–CD31-biotin and anti–VEGFR-2–biotin antibodies were detected with ALP-conjugated streptavidin ABC complex (DakoCytomation), and anti-GFP antibody was detected with Alexa Fluor 488–conjugated goat anti–rabbit IgG (Invitrogen). ALP color was developed as described above.

The stained sections and dishes were observed and photographed with a microscope (IX-70; Olympus) under UV light or with a confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.). In all assays, we used an isotype-matched control Ig as a negative control and confirmed that the signals were specific and were not due to nonspecific background staining.

Cell preparation and flow cytometry. The fetal liver and head region of embryos at embryonic day 12.5 (E12.5) were dissociated by Dispase II (Boehringer) and drawn through a 23G needle. BM cells were collected from the tibiae and femurs, and PB was collected from the axillary vein using standard methods. The mononuclear cells were isolated by centrifugation from total BM cells or PB on Lymphoprep (AXIS-SHIELD PoC AS) according to the manufacturer's instructions. The cell-staining procedure for the flow cytometry was as described previously (10). The mAbs used in immunofluorescence staining were anti-CD45, anti–c-kit, and anti-lineage (a mixture of ter119, Gr-1, Mac-1, B220, CD4, and CD8) antibodies (all purchased from BD Biosciences). All mAbs were purified and conjugated with either FITC, PE, or biotin. Biotinylated antibodies were visualized with PE-conjugated streptavidin or APC-conjugated streptavidin (BD Biosciences). Cells were incubated for 5 min on ice with a 1:100 dilution of CD16/32 (FcγIII/II Receptor [Fcblock]; BD Biosciences) before staining with primary antibody. Staining procedures were as described previously (19). The stained cells were analyzed and sorted by an EPICS flow cytometer (ALTRA; Beckman Coulter).

Cell cultures. 10^4 sorted HSCs from E12.5 fetal liver or the head region were cultured for 14 d on fibronectin-coated dishes (Becton Dickinson) in EBM-2 medium (Clonetics) supplemented with 10 ng/ml VEGF (PeproTech) or 10 ng/ml PDFB-BB (PeproTech). 10^4 HSCs from fetal liver (E12.5) were cocultured with 10^6 cells from the brain (E12.5) in EBM-2 medium separated by a transwell membrane (pore size 0.4 μm; Becton Dickinson) for 14 d. For the coculture, the EBM-2 medium was supplemented with EGM-2-MV-SingleQuots (Clonetics) containing VEGF, IGF-1, EGF, PDGF-BB, and 5% FBS at 37°C in a 5% CO_2 incubator for 14 d.

10^5 sorted PB cells from mice with hind limb ischemia were cultured on fibronectin-coated dishes in EBM-2 medium supplemented with EGM-2-MV-SingleQuots (Clonetics) at 37°C in a 5% CO_2 incubator for 7–28 d. For limiting dilution analysis, isolated $CD11b^{low}$ and $CD11b^{high}$ cells were cultured as described above and seeded in 96-well plates at several dilutions, with 16 replicate wells for each. Wells that did not contain ECs or MCs were confirmed by immunostaining with anti–PECAM-1 or anti-SMA mAbs after 2 wk of culture by counting the positively stained cells. The number of ECs or MCs was determined by using Poisson distribution. Twice a week, one third of the culture medium was removed and replaced with fresh medium containing growth factors.

Mouse model of hind limb ischemia and cell implantation. All surgical procedures were performed as described previously (43). In brief, the recipient mice were anesthetized, and ligation and resection of the left femoral artery and vein were performed. After 3 d, 2×10^5 sorted $CD11b^+$, $CD11b^{low}$, $CD11b^{high}$, or $CD11b^-$ cells from the PB of ischemia-induced green mice were suspended in 50 μl PBS and implanted into the ischemic thigh muscle of mice at five different injection points. 3×10^5 $CD11b^{low}$, $CD11b^{high}$, or $CD11b^-$ cells sorted as described above were suspended in 100 μl PBS and injected intravenously via the tail vein into ischemia-induced C57BL/6 mice. The thigh muscles were dissected out 3 d, 14 d, or 6 mo after transplantation. Tissue staining with anti-GFP (Medical Biological Laboratories Co., Inc.), CD31, CD31-PE (BD Biosciences), and PDGF-β-PE was performed as described above to evaluate the localization of injected GFP^+ cells and the number of ECs and SMCs.

Staining of blood vessels with lectin in ischemic hind limbs. Lectin staining was performed as described previously with some modifications (44). In brief, 3 d after cell transplantation, mice were perfused with 2% paraformaldehyde to fix the tissue. After fixation, blocking was performed with 1% BSA followed with perfusion with biotinylated lectin (20 μg/ml). The muscles were then removed and sectioned at 200 μm by Vibratome 1500 (The Vibratome Company). To localize the lectin binding, samples were incubated at 4°C overnight with streptavidin HRP (DakoCytomation). Color reaction was performed with 0.05% 3,3′-diaminobenzidine (DAB; Wako) and 0.01% H_2O_2 in Tris buffer, pH 7.4, at room temperature. After the color reaction, samples were washed thoroughly with PBS and cleared in BABB solution (benzyl alcohol/benzyl benzoate/methanol; 1:2:1).

Online supplemental material. The online supplemental material contains detailed information regarding the analysis of vascular-specific marker expression in cultured ECs and MCs (Fig. S1), detection of apoptotic cells in cultured ECs (Fig. S2), localization of endogenous $CD11b^+$ cells recruited into ischemic muscle (Fig. S3), analysis of $CD11b^{low}$ cell development from Ang-1 mutant embryos (Fig. S4), in vitro manipulation of $CD11b^{low}$ cells from adult mouse BM (Figs. S5 and S6), in vitro CFU-c analysis (Table S1), and RT-PCR analysis (Table S2). The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050373/DC1.

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