CD31\(^+\) Cells Represent Highly Angiogenic and Vasculogenic Cells in Bone Marrow

Novel Role of Nonendothelial CD31\(^+\) Cells in Neovascularization and Their Therapeutic Effects on Ischemic Vascular Disease

Hyongbum Kim,* Hyun-Jai Cho,* Sung-Whan Kim, Bianling Liu, Yong Jin Choi, JiYoon Lee, Young-Doug Sohn, Min-Young Lee, Mackenzie A. Houge, Young-sup Yoon

**Rationale:** Bone marrow (BM) cells play an important role in physiological and therapeutic neovascularization. However, it remains unclear whether any specific uncultured BM cell populations have higher angiogenic and vasculogenic activities. Moreover, there has been controversy regarding the vasculogenic ability of BM cells.

**Objective:** Preliminary flow cytometric analysis showed that CD31, traditionally a marker for endothelial cells, is expressed in certain nonendothelial BM mononuclear cells in both human and mouse. Based on the conserved CD31 expression in the axis of hematopoietic stem/progenitor cells (HSC/HPCs) to endothelial cells, we further sought to determine the comprehensive vasculogenic and angiogenic characteristics of human and mouse BM-derived CD31\(^+\) cells.

**Methods and Results:** Flow cytometric analysis demonstrated that all CD31\(^+\) cells derived from BM were CD45\(^+\) and expressed markers for both HSC/HPCs and endothelial cells. Comprehensive gene expression analyses revealed that BM-CD31\(^+\) cells expressed higher levels of angiogenic genes than CD31\(^-\) cells. Endothelial progenitor cells, as well as HSC/HPCs, were almost exclusively confined to the CD31\(^+\) cell fraction, and culture of CD31\(^+\) cells under defined conditions gave rise to endothelial cells. Finally, injection of CD31\(^+\) cells into ischemic hindlimb repaired ischemia, increased expression of angiogenic and chemoattractive factors, and, in part, directly contributed to vasculogenesis, as demonstrated by both 3D confocal microscopy and flow cytometry.

**Conclusions:** These data indicate that BM-CD31\(^+\) cells represent highly angiogenic and vasculogenic cells and can be a novel and highly promising source of cells for cell therapy to treat ischemic cardiovascular diseases. (*Circ Res.* 2010;107:602-614.)

**Key Words:** bone marrow ■ CD31 (PECAM-1) angiogenesis ■ vasculogenesis ■ peripheral vascular disease
Interestingly, a majority of the studies reporting the nondifferentiation of EPCs used MI models. Unique conditions of the heart, including the constant motion of the myocardium and the high oxygen consumption of cardiomyocytes make sustained survival of the transplanted cells more difficult. This could reduce the observed differentiation, possibly masking true differentiation potential. Whereas early EPCs are isolated by relatively short-term culture of peripheral blood cells, a newer type of EPCs, known as outgrowing ECs, late EPCs, or endothelial colony-forming cells (ECFCs), can be derived from relatively long-term culture (more than 10 days). Cultured early EPCs and EPC–colony-forming unit (CFU) are indeed angiogenic cells coexpressing myeloid and endothelial markers in contrast to late EPC* or ECFC. Moreover, to date, no markers are unanimously accepted to define uncultured EPCs in circulation or BM in mice or humans.

It now seems that paracrine, or humoral, effects are the main mechanism responsible for the therapeutic effects of BM-derived cell therapy for ischemic cardiovascular disease. Because most of the cells used for previous studies were selected based on their stem cell–like or vasculogenic potential, identification of cells with higher angiogenic or paracrine activities is now necessary to improve therapeutic efficacy and to develop next generation cell therapy for ischemic cardiovascular diseases. However, little research has been conducted to identify cells having higher angiogenic effects. Based on the above concerns and newly developed mechanistic findings, we sought to identify and isolate a specific cell population which possesses higher angiogenic or paracrine activities, includes vasculogenic cells and does not need ex vivo culture for identification or therapeutic use. In pursuit of this goal, we found that CD31 appears to be a good marker for this purpose. CD31 (also known as platelet endothelial cell adhesion molecule-1) is a 130-kDa, type I transmembrane glycoprotein, and is a member of the immunoglobulin gene superfamily. CD31 is highly expressed on ECs and to different degrees on several hematopoietic cells, including monocytes, granulocytes, and platelets. Furthermore, CD31 is expressed in lineage* Sca-1–c-Kit+ (LSK) mouse HSCs, side population cells in mouse BM, mouse embryonic stem cells, and human early EPCs. However, the expression of CD31 in mouse hematopoietic progenitor cells (HPCs) and human HSCs has yet to be clearly investigated. Interestingly, HSCs/HPCs were already known to have angiogenic and/or vasculogenic potential as well as favorable cardiovascular regenerative effects. This conserved expression of CD31 in the axis of HSC/HPCs, EPCs and ECs has led us to investigate whether this group includes all the necessary cells associated with neovascularization and may be beneficial for treating ischemic cardiovascular diseases.

Methods

Animals
C57BL/6J mice, GFP-transgenic mice (C57BL/6J background) and athymic nude mice (The Jackson Laboratory) were used. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of Caritas St. Elizabeth Medical Center and Emory University.

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Harvesting BM-Mononuclear Cells and CD31 Selection

Human BM (hBM) from healthy adults was purchased from Lonza, and mouse BM (mBM) cells were harvested as previously described. Mouse and human BM-mononuclear cells (MNCs) were isolated by density gradient centrifugation using Histopaque 1083 (Sigma) and Histopaque 1077 (Sigma), respectively. Mouse and human BM-CD31+ and CD31– cells were isolated using magnetic columns (MACS, Miltenyi Biotec).

Transplantation of the Cells Into Ischemic Hindlimb

Hindlimb ischemia was generated by ligation of the femoral artery and catherization of large branches. One million mBM-CD31+ or mBM-CD31– cells in 100 µL PBS were intramuscularly injected into the ischemic hindlimb of C57BL/6J mice, and cells in 200 µL PBS were intravenously injected into nude mice with ischemic hindlimb.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes information about the following items: microarray and gene set enrichment analysis, colony forming unit assay, BM transplantation, EPC culture assay, induction of differentiation of CD31+ cells into ECs in vitro, blood flow measurement in ischemic hindlimbs, measurement of capillary density, histological analysis, cell adhesion test, and real-time RT-PCR.

Results

Dual Hematopoietic and Endothelial Cell Characteristics of BM-CD31+ Cells

To characterize the phenotype of mBM-CD31+ cells, we first isolated mBM-MNCs from C57BL/6J mice and carried out flow cytometric analysis (Figure 1A). ~34% of mBM-MNCs were found to express CD31. Next, we determined which subsets of mBM-MNCs express CD31 using antibodies specific to B- and T-lymphocytes, myelomonocytic cells, and erythroid cells. FACs analysis showed that, on average, CD31 was expressed in 73% of lineage-depleted (Lin-) cells, 53% of B- (B220+) lymphocytes, 42% of T- (CD3e+) lymphocytes, 12% of erythroid cells (Ter-119+), 17% of CD11b+ myelomonocytic cells and 17% of Gr-1+ my-
elomonocytic cells (Figure 1B). Among myelomonocytic cells which express CD11b or Gr-1, the rate of CD31 expression was higher in Ly-6g- cells (mainly monocytes, 32%, data not shown) than in Ly-6g+ cells (mainly granulocytes, 4.4%, Figure 1B). Among CD31+ cells, 5% were Lin- cells, and 63% expressed B-220, 10% CD3e+, 2% Ter-119+, 28% CD11b+, and 29% Gr-1+, respectively (Figure 1C). Virtually all (>99%) mBM-CD31+ cells expressed CD45 (Figure 1C), suggesting that the CD31+ cells are hematopoietic cells, but not ECs, existing in BM. Among myelomonocytic CD31+ cells (CD11b- or Gr-1-expressing CD31+ cells), only 9% were Ly-6g+ (Figure 1D), suggesting that a majority of myelomonocytic CD31+ cells are monocytes.

Next, we explored whether mBM-MNCs express other endothelial cell markers, and if so, to determine whether these cells express higher levels of CD31, thereby representing more highly angiogenic or vasculogenic cells. FACS analysis showed that mBM-MNCs which expressed various EC markers also coexpressed CD31 at the following rates: VE-cadherin, 77%, Flk-1 (VEGFR-2), 63%, Tie2, 76% and von Willebrand factor (vWF), 87% (Figure 1E). Triple staining with CD45 antibody, a pan-hematopoietic cell marker, showed that the majority of cells double-positive for CD31 and any of the EC markers also expressed CD45 (Figure 1F), suggesting that these cells are hematopoietic in origin rather than circulating mature ECs. Human BM-derived CD31+ (hBM-CD31+) cells also had similar hematopoietic and endothelial characteristics. FACS analysis showed that ∼35% to 50% of hBM-MNCs expressed CD31 (Online Figure I, A), and that CD31 was expressed in 42% of CD19+ cells (B cells), 46% of CD3+ cells (T cells), 67% of CD14+ cells, and 76% of CD11b+ cells (Online Figure I, B). Among CD31+ cells, 19% expressed CD19, and 39%, 33%, and 29% were CD3+, CD14+, and CD11b+, respectively (Online Figure I, C). Similar to mBM-CD31+ cells, almost all hBM-CD31+ cells expressed CD45 (Online Figure I, C), indicating their hematopoietic, but not EC, identity. Again, similar to the mouse data, a majority of hBM cells expressing EC markers expressed CD31: 90% in VE-cadherin+ BM cells, 65% in KDR+ cells, 55% of Tie2+ cells, and 96% in vWF+ cells (Online Figure I, D). As in the mouse, most of the human BM cells double-positive for CD31 and any of the EC markers expressed CD45 (Online Figure I, E), indicating hematopoietic origin.
BM-CD31+ Cells Are Enriched With Higher Levels of Angiogenic and HSC/HPC Genes

To determine whether mBM-CD31+ cells are enriched with angiogenic and hematopoietic stem/progenitor genes, we carried out microarray analysis. We compared the gene expression among CD31+, CD31-, Lin-CD31+, and Lin-CD31- cells using microarray and gene set enrichment analysis (GSEA) to determine the enriched gene classes in each population (Figure 2). The GSEA showed that proangiogenic, but not antiangiogenic genes were significantly upregulated in mBM-CD31+ cells as compared to mBM-CD31- cells (Figure 2A). Furthermore, the expression levels of HSC/HPC-specific genes were also significantly higher in mBM-CD31+ cells than in mBM-CD31- cells. Higher expression of proangiogenic genes and HSC/HPC genes was also observed in Lin-CD31+ cells compared to Lin-CD31- cells. These data suggest that CD31 is a marker for angiogenic cells and HSCs/HPCs in both total BM cells and Lin-BM cells. Next, we further carried out leading-edge subset analysis to identify the core proangiogenic genes that confer angiogenic characteristics of CD31+ cells, and found that high expression levels of 22 proangiogenic genes comprised the angiogenic phenotype of mBM-CD31+ cells (Figure 2B). The higher expression levels of most of these genes were also confirmed by real-time RT-PCR analysis (Figure 2B).

Similar experiments were performed for human BM-CD31+ and CD31- cells. GSEA showed significantly higher expression of proangiogenic genes and HSC/HPC genes in hBM-CD31+ cells as compared to hBM-CD31- cells (Figure 2C and 2D), indicating that angiogenic cells and HSC/HPCs are enriched in the CD31+ fraction. The leading-edge subset analysis identified high expression levels of 24 core proangiogenic genes, including VEGF-A and angiopoietin-1, in hBM-CD31+ cells.

HSCs and HPCs Are Almost Exclusively Restricted to the BM-CD31+ Cell Population

We explored the expression of CD31 in HSCs and HPCs. Approximately 91% of LSK cells in mouse BM, which include HSCs and multipotent progenitor cells (MPPs), express CD31 (Figure 3A). Because it appears that the level of CD31 expression decreases as HSCs undergo differentiation into mature hematopoietic cells, we examined the expression of CD31 in cells of intermediate status, ie, oligopotent progenitor cells such as common lymphocyte progenitors.
(CLPs) and common myeloid progenitors (CMPs). As in LSK cells, most CLPs and CMPs identified as Lin−IL-7Ra+ c-Kitlow Sca-1 low Thy-1.1−28 and Lin−IL-7Ra−c-Kit+ Sca-1+ FcγRlow CD34+ cells, respectively, expressed CD31 (Figure 3A), suggesting that CD31 expression is maintained from HSCs and MPPs to oligopotent progenitor cells. To confirm that CD31+ cells include HSCs and HPCs, we performed an in vitro clonogenic assay and an in vivo BMT experiment. As expected, colony forming units, including BFU-E, CFU-GM, and CFU-GEMM, were almost exclusively observed among mBM-CD31+ cells (Figure 3B and 3C). Transplantation of 1×10^5 mBM-CD31+ cells into lethally irradiated mice rescued all of the mice (10/10), whereas transplantation of mBM-CD31− cells saved only 20% of the mice (2/10) (Figure 3D). In experiments with human BM-MNCs, we found that cells expressing CD34 and CD133 were enriched in the CD31+ fraction (Online Figure II, A). Furthermore, CD31 was expressed in 99.8% of CD34+CD133− cells and 89% of CD34+CD133+ cells (Online Figure II, B), suggesting that HSCs and most HPCs are included in the CD31+ cell population. Taken together, these results indicate that HSCs and HPCs in both mice and humans exist almost exclusively in the CD31+ fraction.

EPCs Are Cultured From the CD31+, But Not CD31−, Cell Fraction

We determined whether mBM-CD31+ cells show endothelial differentiation potential in vitro. First, conventional early
EPC culture conditions were applied to CD31+ and CD31− cells, and EPCs were identified by Dil-ac-LDL uptake and binding to BS-1 lectin.1,30 Surprisingly, such EPCs were exclusively cultured from mBM-CD31+ cells, but not from mBM-CD31− cells (Figure 4A and 4B). When the CD31+ cells were cultured for 8 or 9 days in EGM-2 media supplemented with additional VEGF (50 ng/mL), the adherent cells expressed vWF, Fk-1, VE-cadherin, and CD31, indicating an EC phenotype (Figure 4C). Similarly, the early EPC assay using human BM cells showed that EPCs were cultured almost exclusively from hBM-CD31+, but not hBM-CD31− cells (Online Figure III, A). When hBM-CD31+ cells were cultured in EGM-2 with 20% FBS for 6 days, the cells also underwent EC phenotypic changes such that a majority of cells expressed endothelial-specific proteins such as vWF, KDR, VE-cadherin, and CD31 (Online Figure III, B). The CFU-Hill assay27 also showed that CFU-Hill colonies were higher in hBM-CD31+ cells than hBM-CD31− cells (Online Figure III, C).

**BM-CD31+ Cells Improve Hindlimb Ischemia and Increase Vascularity**

To examine the therapeutic effects and vascularizing capacity of mBM-CD31+ cells in vivo, we intramuscularly transplanted mBM-CD31+ cells into mice in a hindlimb ischemia model. Nude mice were initially used, because 60% to 100% of nude mice lose all or part of their hindlimbs following surgery, providing unambiguous results on the therapeutic efficacy of transplanted cells.17 In contrast, C57BL/6 mice spontaneously recover their blood flow to near normal levels within 4 weeks, making it difficult to evaluate the therapeutic effects of the treatment. mBM-CD31+ cells injected into nude mice prevented limb loss in 6 of 7 mice (86%), whereas mBM-CD31− cells and PBS treatment rescued limbs in only 1 of 7 and 0 of 7 mice, respectively (Figure 5A). LDPI analysis also revealed that mBM-CD31+ cell transplantation resulted in 1.8- and 2.4-fold enhanced blood flow compared to mBM-CD31− cells and PBS-treated controls, respectively (Figure 5C and 5D; mBM-CD31+ versus mBM-CD31− and PBS, 33±2 versus 43±7 and 79±5%, P<0.01). We tested the therapeutic effects again in C57BL/6 mice by injecting cells intramuscularly to increase the engraftment. Again, mBM-CD31+, but not mBM-CD31− cell-injected mice showed significantly improved perfusion in the hindlimb at day 14 compared to the PBS treated mice (P<0.01) (Online Figure IV, A and B). In addition, injection of human BM-CD31+ cells into nude mice with hindlimb ischemia showed similar therapeutic effects represented by higher limb salvage at 3 weeks (Online Figure V, A and B) and 1.5- to 2.4-fold higher blood flow in the hindlimb over 1 to 3 weeks than hBM-CD31− cells and PBS (Online Figure V, C and D).

We also compared the therapeutic effects of the CD31+ cells with well characterized other BM cell subpopulations on the recovery of hindlimb ischemia and found that there was no difference in the capability to recover hindlimb ischemia among the CD31+ cells, CD34+ cells,18,24 CD34−CD31+ cells, and cultured ECs.10,17 (Online Figure VI). The therapeutic effects of CD31+ cells were significantly higher compared to total mononuclear cells (Online Figure VII). To determine the therapeutic effects of HSC/HPCs included in CD31+ cells, mouse Lin−CD31+ cells and Lin−CD31− cells were compared. The therapeutic effects of these 2 subpopulations was not significantly different (Online Figure VIII), suggesting that the therapeutic effects of CD31+ cells are attributable to both subpopulations. To determine whether the CD31+ cells are still effective in subjects having cardiovascular risk factors, we tested the therapeutic effect of the CD31+ cells isolated from apolipoprotein E knockout (ApoE-KO) mice fed a high-fat diet, a well known atherosclerosis model. We found no difference in the therapeutic effects between the CD31+ cells derived from normal mice and ApoE-KO mice, whereas the effects of CD31− cells derived from ApoE-KO mice were significantly lower compared to CD31+ cells derived from normal or ApoE-KO mice (Online Figure IX).

To determine the effects of the CD31+ cells on neovascularization, we counted the number of capillaries in the hindlimb muscles. mBM-CD31+ cell-injected nude mice showed 2.2- or 2.3-fold higher capillary density than mBM-CD31− and PBS-injected mice, respectively (CD31+ versus CD31, PBS; 947±114 versus 436±53, 408±47 capillaries/mm², P<0.05) (Figure 5E and 5F). The beneficial effects of mBM-CD31+ cell injection on capillary density were also observed in C57BL/6 mice but with less prominence (Online
BM-CD31⁺ Cells Have Higher Engraftment Potential Than BM-CD31⁻ Cells

We examined the engraftment of the transplanted cells into ischemic muscles. For this, we isolated CD3₁⁺ and CD3₁⁻ cells from GFP-transgenic mice and injected them into a hindlimb ischemia model. Fluorescent microscopic examination showed that 2 weeks after cell injection, GFP-positive cells were more abundant in the CD3₁⁺ group than in the CD3₁⁻ group (Figure 6A; Online Figure XI; Online Videos 1 and 2). To quantify the number of engrafted cells, in a separate series of experiments, we enzymatically digested the hindlimb muscles and determined the number of GFP-positive cells by flow cytometric analysis. This analysis showed that the number of engrafted cells was 3.4-fold higher in the CD3₁⁺ group than in the CD3₁⁻ group (P<0.05) (Figure 6B). To further investigate the mechanism underlying this higher engraftment of mBM-CD3₁⁺ cells, we carried out in vitro adhesion studies, in which we investigated the adhesion capability of the cells to representative extracellular matrix (ECM) proteins of the muscle tissue. This adhesion assay revealed that mBM-CD3₁⁺ cells displayed 5-, 10-, and 2-fold higher adhesion to collagen, laminin, and vitronectin, respectively, compared to the mBM-CD3₁⁻ cells (Figure 6C and 6D), indicating that higher adhesion capacity may underlie the higher engraftment of mBM-CD3₁⁺ in vivo.

BM-CD31⁺ Cells Induce Vasculogenesis In Vivo

We determined the vasculogenic potential of mBM-CD31⁺ cells in vivo, ie, whether the transplanted CD3₁⁺ cells can give rise to ECs. As the vasculogenesis of BM cells is disputed, we used rigorous criteria to judge the incorporation of cells into blood vessels as ECs. To track the transplanted cells, we isolated CD3₁⁺ and CD3₁⁻ cells from the BM of GFP-transgenic mice (Figure 7) and confirmed these findings again with Dil-labeled cells (Online Figure XII). To identify functional ECs, we intravenously injected ILB4, which stains the luminal side of ECs in functioning blood vessels.⁹ We
also used high resolution confocal microscopy and analyzed the images by Z-stacked 3D reconstruction and FACS analysis. The images showed that mBM-CD31\(^+\)/H11001 cells were incorporated into functional capillaries and displayed typical EC morphology (Figure 7A and 7B; Online Video 3), indicating true vasculogenic potential of mBM-CD31\(^+\)/H11001 cells. Then, we quantified the number of vasculogenic cells derived from the transplanted mBM-CD31\(^+\)/H11001 cells using flow cytometric analysis after enzymatic digestion of the hindlimb tissues.\(^9\) ECs were identified by in vivo perfusion of ILB4 and the transplanted cells were identified by their intrinsic GFP fluorescence. The number of injected cells exhibiting ILB4 was 3.7-fold higher in the CD31\(^+\) group than in the CD31\(^-\) group (engrafted cells/total ILB4 ECs, mBM-CD31\(^+\)/H11001 versus mBM-CD31\(^-\), 3.3\(\pm\)0.8 versus 0.9\(\pm\)0.3%, \(P<0.05\)) (Figure 7C and 7D), suggesting that vasculogenic cells are significantly enriched in the CD31\(^+\) population. As

![Figure 6. Higher engraftment and adhesion capacity of mBM-CD31\(^+\) cells. A and B, Microscopic and FACS analyses of the engrafted cells. The CD31\(^+\) and CD31\(^-\) cells isolated from GFP mice were intramuscularly injected into ischemic hindlimbs. Two weeks later, the engrafted cells in the hindlimbs were examined by confocal microscopic (A) and flow cytometric (B) analysis (\(*P<0.05, n=5\)). C and D, Representative images and quantitative analysis of the adhesion assay. CD31\(^+\) and CD31\(^-\) cells were plated onto dishes with various ECM proteins and the number of adherent cells was determined by fluorescent microscopy (\(*P<0.05, n=3 \text{ to } 5\)). Bars: 50 \(\mu\)m (A); 100 \(\mu\)m (C).](https://circres.ahajournals.org/doi/fig/10.1161/01.CIR.0000460053.03269.3d)
CD31\(^+\) cells include HSCs, HPCs, and a fraction of myelomonocytic cells, all of which were reported to have vasculogenic effects, we further investigated what fractions contribute to the vasculogenic effect of the CD31\(^+\) cells. For this, we quantitatively evaluated the vasculogenic effect of CD34\(^+\) cells (HSCs and HPCs) and CD14\(^+\) cells (myelomonocytic cells) with CD34-CD31\(^+\) cells and CD14-CD31\(^+\) cells, respectively. The vasculogenic effects of CD34\(^+\) and CD34\(^+\)CD31\(^+\) cells were similar (Online Figure XIII, A and B), suggesting that the CD34\(^+\)CD31\(^+\) cell fraction also contains a significant number of vasculogenic cells. Intriguingly, the vasculogenic effect of CD14\(^+\)CD31\(^+\) cells were significantly higher than CD14\(^+\) cells (Online Figure XIII, C and D), indicating that a considerable portion of vasculogenic cells are enriched in nonmyelomonocytic CD31\(^+\) cells. Together these results suggest that CD31\(^+\) cells include novel vasculogenic cells that do not belong to known vasculogenic populations: hematopoietic stem and progenitor cells (CD34) and myelomonocytic cells (CD14).

Figure 7. Vasculogenesis in the ischemic hindlimb tissue. mBM-CD31\(^+\) or -CD31\(^-\) cells were isolated from GFP mice and IM injected into ischemic hindlimbs. One (A and B) or 2 weeks (C and D) after the transplantation, mice were IV injected with Alexa 647-conjugated ILB4. A and B, Representative confocal images. Blue, DAPI (nuclei); green, GFP (CD31\(^+\) cells); red, ILB4 staining. A and B, Orthogonal (A) and 3D-projection (B) views with corresponding single color images. Transplanted CD31\(^+\) cells incorporated into functional blood vessels as ECs. C, Representative FACS analysis of digested hindlimb tissue to determine ECs derived from the transplanted cells. D, The number of ECs derived from injected cells. (\(P<0.05, n=5\)). Bars, 10 \(\mu\)m (A and B).
Transplantation of BM-CD31+ Cells Upregulates Angiogenic, Antiapoptotic, and Chemoattractant Factors in Ischemic Hindlimbs

To investigate the effects of mBM-CD31+ cells transplanted into ischemic hindlimb, the biological factors associated with neovascularization were measured by real-time RT-PCR on adductor muscles harvested 1 week after treatment. Various angiogenic factors, such as Fgf2, Plgf, Angpt1, Angpt2, Tgfb, and Il6, were more highly expressed in the mBM-CD31+ group compared to the CD31/H11001 and PBS groups (Figure 8). Interestingly, Angpt2 increased by 400 fold, whereas Angpt1 increased by only 13 fold in the CD31/H11001 group compared to the PBS group. The relative predominance of Angpt2 over Angpt1 has been previously reported to be an indicator of a proangiogenic milieu.31 Furthermore, antiapoptotic and angiogenic factor Igf1 was significantly upregulated in the CD31/H11001 group compared to the other groups. A representative chemoattractant molecule, Cxcl12 (Sdf1a) was also significantly upregulated in the CD31+ group compared to the other groups. Taken together, these findings indicate that many representative biological factors associated with angiogenesis, antiapoptosis, and BM cell chemotaxis were significantly upregulated in the ischemic muscles injected with mBM-CD31+ cells, supporting a paracrine role for CD31+ cells in the improvement of hindlimb ischemia.

Discussion

In this study, we report that CD31 is a unique marker of a full spectrum of nonendothelial hematopoietic BM cells that are tightly associated with neovascularization, as evidenced by high angiogenic properties, high adhesion capacity, exclusive HSC/HPC activity, and vasculogenic ability. These versatile functions enable CD31+ cells to induce higher therapeutic neovascularization and greater therapeutic effects on the repair of limb ischemia.

CD31+ cells are highly proangiogenic cells both in mouse and human BM. To our knowledge, this is the first study to demonstrate, with the use of multiple objective analyses, that a specific, nonmanipulated cell population in BM is more highly angiogenic than others. In GSEA analysis, we used a priori-defined gene sets to analyze the data because post hoc examination of top-ranking genes can be subjective and prone to bias. The histological analysis of hindlimb tissues showed that incorporation of transplanted CD31+ cells into the vasculature as ECs was significant, but accounted for only 3.3% of the total functional ECs. However, CD31+ cell injection increased capillary density by 1.3-fold, implying that nonvasculogenic effects of the transplanted CD31+ cells are a more dominant mechanism for neovascularization. Compatible with these data, transplantation of CD31+ cells resulted in global upregulation of multiple angiogenic, antiapoptotic, and chemotactic factors in the ischemic limb.

With regard to the vasculogenesis of BM cells, we provide unambiguous evidence for the physical contribution of injected BM cells to endothelial cell generation. Some recent studies have argued against the vasculogenic potential of externally injected BM cells, particularly in cardiovascular models.11 We assumed that much of this controversy originated from the technical difficulties of confirming vasculogenesis or from the difference in the experimental settings...
such as type of injected cells, the recipient environment (animal models), or route of cell administration. Supporting our notion is the fact that the data regarding the absence of differentiation of BM cells into endothelial cells and cardiomyocytes came from studies using specific HSC populations and specific animal models such as MI, in which long-term engraftment and survival of transplanted cells is expected to be low because of constant motion of the heart and little oxygen and nutrients available for the transplanted cells. In fact, another study refuting the transdifferentiation of similar HSCs to cardiomyocytes did not mention the potential of endothelial transdifferentiation of the same cells. Other recent studies showing the direct contribution of BM cells to new vasculature used several tumor models and BM transplantation. Based on these observations, we hypothesized that postnatal vasculogenesis can occur through BM cells, although it may not occur frequently and may not be the main cause of the therapeutic effects of BM cells on cardiovascular disease. With the following extensive and meticulous analyses, the use of both 3D reconstruction of confocal images, flow cytometric analysis of the digested tissues, use of both chemical dye and GFP-transgenic mice for cell targeting, and application of rigorous criteria, we were able to confirm that BM cells, especially CD31+ cells, can generate significant numbers of ECs in ischemic tissues. Thus, we were able to conclude that when an appropriate BM cell is introduced into a specific environment (here, hindlimb ischemia), genuine vasculogenesis from BM cells can occur. This idea can be further expanded to describe the differentiation into ECs as a context-dependent phenomenon rather than an “all or none” event. This study provides convincing evidence for the occurrence of vasculogenesis or endothelial differentiation by externally injected BM cells in at least an ischemic vascular disease setting.

This study further revealed another novel mechanism underlying the observed therapeutic effect, cell engraftment. The higher paracrine and vasculogenic effects of CD31+ cells may be, at least in part, attributable to higher engraftment of the transplanted CD31+ cells. The mechanisms underlying the higher engraftment of CD31+ cells may be stronger adhesion of CD31+ cells to the ECM, which prevents the transplanted cells from both anoikis (apoptosis caused by lack of adhesion to ECMs) and cell removal by the vascular system. The importance of cell engraftment and cell survival for increasing therapeutic effects has been demonstrated by previous studies which used overexpression of the Akt pathway in transplanted cells or application of heat shock before transplantation. This study demonstrates, for the first time, that a certain native population of BM cells, CD31+ cells, possesses higher engraftment capability on transplantation. We also do not exclude possible favorable effects of an immunomodulatory role of the CD31+ cells on therapeutic effects. Given that CD31+ cells include B and T lymphocytes and other potential immune related cells, CD31+ cells may exert an immunomodulatory function to reduce tissue damage and augment tissue regeneration. We also showed that mouse and human HSCs and HPCs are almost exclusively restricted to the CD31+ fraction. Although mouse HSCs have been reported to express CD31, it was not known whether human HSCs and mouse HPCs do so as well. Our flow cytometric analysis, in vitro clonogenic assay, and short-term BM transplantation studies demonstrated that almost all HSCs and HPCs both in mouse and human BM are CD31+, and we propose CD31 to be a universal marker for HSCs and HPCs. Because there have been reports showing the favorable effects of HSCs for ischemic cardiovascular diseases, the enrichment of HSCs in the CD31+ fraction may provide another advantage of using CD31+ cells for therapeutic purposes.

Our study provides compelling evidence for the therapeutic utility of CD31+ cells for regenerating ischemic tissues. In earlier studies, the criteria to select a specific population for therapeutic use was based on the 2 premises that HSC/HPCs would have higher differentiation potential to give rise to ECs, and that therapeutic effects are mainly attributable to the vasculogenic effect of the transplanted cells. Thus, HSC/HPC or EPC markers, such as Lin−c-kit−, CD34+, CD133+, and Hoechst dye exclusion (side population), have been used to select therapeutically effective cells. However, given the recent reports that therapeutic effects of BM cells in ischemia repair are mainly attributable to humoral or angiogenic effects, isolating more highly angiogenic and/or paracrine cells may be a better option for cell therapy. In this respect, BM-CD31+ cells could serve as a favored option for second-generation cell therapy, as CD31+ cells have several advantages over other cell types. First, transplantable CD31+ cells can be prepared without ex vivo culture, which is required for EPCs and mesenchymal stem cells. Second, CD31+ cells are relatively abundant. Unlike CD133+ and CD34+ cells, it is unnecessary to collect a large volume of BM or to mobilize BM cells. Third, CD31+ cells are safer; we did not observe any of the adverse effects, such as aggravation of hindlimb ischemia, calcification, or tumor formation, reported in the use of BM-MNCs, whole BM cells, or mesenchymal stem cells, respectively. However, given the recent reports showing a contribution of CD31 to the development of atherosclerosis in abdominal aorta in an animal model, close monitoring is required when using CD31+ cells in atherosclerotic disease. Another concern is the effects of the disease state on the function of CD31+ cells. Although CD31+ cells derived from ApoE KO mice are therapeutically comparable to wild-type CD31+ cells, we do not exclude the possibility that CD31+ cells derived from chronically ill patients with multiple cardiovascular risk factors may be less efficacious. Together, this study suggests that CD31− cells could serve as a novel therapeutic option for treating ischemic vascular diseases.

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Disclosures

None.

References


What Is Known?

- Bone marrow (BM)-derived endothelial progenitor cells (EPCs), which are cultured cells, were reported to have blood vessel–growing capability.
- There has been controversy regarding the vasculogenic ability or direct (trans)differentiation of BM-derived cells.
- CD31 is a traditional marker for endothelial cells and is also expressed in a fraction of BM cells.

What New Information Does This Article Contribute?

- CD31 is a comprehensive marker to identify highly angiogenic and vasculogenic cells among uncultured heterogeneous BM mononuclear cells (MNCs) both in human and mouse.
- A fraction of BM-MNCs that express CD31 can contribute to blood vessel formation by differentiation into endothelial cells.
- Human and mouse BM-derived CD31+ cell transplantation ameliorated the effects of ischemia in a hindlimb ischemia model.

Cell therapy with BM cells has emerged as a new therapeutic option for treatment of ischemic cardiovascular diseases. However, several important questions remain regarding the therapeutic mechanism and choice of cells: whether BM cells directly contribute to the generation of endothelial cells (vasculogenesis) and whether there is a marker to identify cells possessing high blood vessel forming capabilities among uncultured heterogeneous BM cells. In this study, we show for the first time that CD31 can serve as a marker to identify highly angiogenic and vasculogenic cells among uncultured BM-MNCs. We also provide evidence for the occurrence of adult vasculogenesis from BM-CD31+ cells injected into ischemic tissue. We discovered that CD31+ cells showed higher adhesion and engraftment properties and paracrine activities than CD31− cells and are effective for treating ischemic vascular disease; however, the major therapeutic mechanism(s) underlying the effects of CD31+ cells is independent of endothelial differentiation of the transplanted cells. This study suggests that BM-CD31+ cells can be a highly promising cell source for cell therapy to treat ischemic cardiovascular diseases.
CD31+ Cells Represent Highly Angiogenic and Vasculogenic Cells in Bone Marrow: Novel Role of Nonendothelial CD31+ Cells in Neovascularization and Their Therapeutic Effects on Ischemic Vascular Disease

Hyongbum Kim, Hyun-Jai Cho, Sung-Whan Kim, Bianling Liu, Yong Jin Choi, JiYoon Lee, Young-Doug Sohn, Min-Young Lee, Mackenzie A. Houge and Young-sup Yoon

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SUPPLEMENTAL MATERIALS

Hyongbum Kim, Hyun-Jai Cho, Sung-Whan Kim, Bianling Liu, Yong Jin Choi, Ji Yoon Lee, Young-Doug Sohn, Min-Young Lee, Mackenzie A. Houge, Young-sup Yoon

CD31⁺ cells represent highly angiogenic and vasculogenic cells in bone marrow: novel role of non-endothelial CD31⁺ cells in neovascularization and their therapeutic effects on tissue ischemia
Expanded Materials and Methods

Animals
Wild-type C57BL/6J mice, GFP-transgenic mice (C57BL/6J background, Enhanced GFP expression is under the control of the β-actin promoter) and athymic nude mice (The Jackson Laboratory) aged 8 – 12 week were used. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of Caritas St. Elizabeth Medical Center and Emory University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Harvesting BM-mononuclear cells and CD31 selection
Human BM from healthy adults was purchased from Lonza, and mouse BM cells were harvested as previously described 1. Mouse and human BM-MNCs were isolated by density gradient centrifugation using Histopaque 1083 (Sigma) and Histopaque 1077 (Sigma), respectively. Mouse BM-MNCs were stained with biotinylated anti-mouse-CD31 monoclonal antibody (BD Biosciences) for 30 min at 4°C, washed, and incubated with streptavidin magnetic beads (Mitenyi Biotec, Germany) for 20 min at 4°C. Human BM-MNCs were labeled with anti-human CD31 antibody conjugated to magnetic beads (Milenyi Biotec). Mouse and human BM-CD31⁺ and CD31⁻ cells were isolated using magnetic columns (MACS®, Miltenyi Biotec) according to manufacturer’s instructions.

Microarray and gene set enrichment analysis
Total RNA was isolated from BM-CD31⁺, BM-CD31⁻, LinCD31⁺, and LinCD31⁻ cells from 8 week old C57BL/6J mice and human BM-CD31⁺ and BM-CD31⁻ cells. Microarray analysis was performed using GeneChip® Mouse Genome 430A 2.0 (Affymetrix, Inc. Santa Clara, CA) and Human Gene 1.0 ST array (Affymetrix). Gene set enrichment analysis (GSEA) was performed as previously described 2 using a GSEA program (http://www.broad.mit.edu/gsea/msigdb/index.jsp). Pro- and anti-angiogenic gene sets, consisting of 108 pro-angiogenic and 44 anti-angiogenic genes, respectively, were collected based on the GO data base (http://www.geneontology.org/) and extensive literature review (Online Table I). For GSEA, we used total 5454 gene sets, which are a combination of C1-C5 gene sets (http://www.broad.mit.edu/gsea/msigdb/collections.jsp) and our pro- and anti-angiogenic gene sets. P < 0.01 and Q < 0.15 was considered statistically significant. The level of pro-angiogenic genes that constitute the leading edge was confirmed by real-time PCR using custom-designed low density array (Applied Biosystems) according to the manufacturer’s instructions.

Flow cytometry
For the analysis of cells in the hindlimb, mice were anesthetized using tribromoethanol (Avertin) and intravenously injected with Alexa-647-conjugated isolecitin B4 (Invitrogen) to specifically stain ECs in vivo as previously described 3. Ten minutes after injection, mice were perfused with PBS to remove blood cells and unbound isolecitin B4 within the blood vessels. The cell-injected area of adductors were removed, minced, and digested at 37°C for 60 – 90 min with an enzyme cocktail (collagenase, elastase, and DNase I (Roche Applied Science)) and single-cell suspensions were prepared by filtering through a 30-μm strainer. BM-MNCs were isolated as described above for analysis. All antibodies used were from BD Biosciences unless otherwise specified. Mouse single-cell suspensions were stained with CD31 (FITC), B220 (PE-Cy7), CD3e (PE), CD11b (APC), Ly-6G (PE), Gr-1 (APC-Cy7), Ter-119 (PE), Linage cocktail (APC), Thy-1.1 (PE-Cy7, eBioscience, Inc., San Diego, CA), CD34 (Alexa 700), CD127 (IL-7Ra, pacific blue, eBioscience), Fc⁺RII/III (PerCP-Cy5.5, eBioscience), CD45 (pacific blue), Tie2 (PE, eBioscience), CD144 (APC, eBioscience), Flk-1 (PE, eBioscience), CD45 (pacific blue, eBioscience), vWF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, with PE-conjugated
secondary antibody), c-Kit (APC-Alexa750, Caltag), MECA32 (with biotinylated secondary antibody and streptavidin-Alexa 647) or Sca-1 (PE) antibodies, and analyzed using the BD LSRII system (BD Biosciences) or the Accuri C6 Flow Cytometer® (Accuri Cytometers Inc., Ann Arbor, MI). Human cells were stained using antibodies against CD31 (FITC or PE), CD19 (APC), CD3 (PE-Cy7), CD14 (PE), CD11b (PE), VE-cad (APC, eBioscience), KDR (PE, R&D systems), Tie2 (PE, R&D systems), vWF (with PE-conjugated secondary antibody), CD34 (APC), or CD133 (PE, Miltenyi Biotec, Germany). Flow cytometric data were analyzed with FlowJo (Tree Star, Inc., Ashland, OR) or CFlow Plus (Accuri Cytometers Inc.) using appropriate controls with isotype-matched IgG and unstained controls.

**Colony forming unit assay**

Cells were cultured in methylcellulose media (Methocult GF M3534 media, Stem Cell Technologies) at a density of 2 x 10^5 cells/ml and the number of colonies was determined after 10 days according to the manufacturer’s instructions.

**BM transplantation**

C57BL/6J mice (female, 6-8 weeks, Jackson Laboratories) were lethally irradiated with 12 Gy in 2 equal doses of 6 Gy delivered 3 hours apart and intravenously injected with 1 x 10^6 mBM-CD31^+ or -CD31^+ cells from GFP transgenic mice into the tail vein. Antibiotic water was provided for 1 month after transplantation and survival was monitored daily for 47 days.

**EPC culture assay**

For mouse early EPC culture assay, mBM-CD31^+ , mBM-CD31^- cells, and mBM-MNCs were plated onto vitronectin-coated 2-well chamber slides (2 x 10^5 cells/slide) and cultured in EGM-2 (EBM-2 media with supplements, SingleQuots except hydrocortisone) (Clonetics) according to the manufacturer’s instructions. At day 4, attached cells were incubated with Dil-acLDL (Biomedical technologies) for 1 hour at 37°C, then washed, fixed in 1% paraformaldehyde, and stained with FITC-conjugated BS-1 lectin (Bandeiraea simplicifolia lectin I, Vector Laboratories) and DAPI. Triple-positive cells for Dil-acLDL, BS-1 lectin and DAPI were counted as EPCs as previous reported 4,5 using an image analyzer (Image-Pro Plus®).

For human early EPC culture, hBM-CD31^+ and hBM-CD31^- cells were plated onto fibronectin/gelatin-coated plates (1 x 10^6 cells/cm^2) and cultured in EGM-2. At day 4, cells were stained as described above but without DAPI. Cells double-positive for Dil-acLDL and UEA-1 lectin were counted as EPCs as described 4,5. The colony-forming unit-Hill (CFU-Hill) assay was performed as previously described 6. Briefly, hBM-CD31^+ or hBM-CD31^- cells were plated in the 6-well fibronectin-coated plate at 5 million cells per well in growth medium consisting of Medium 199 supplemented with 20% FBS and 1% antibiotics (Invitrogen). After 48 hours, the nonadherent cells were collected and re-plated onto a fibronectin-coated 24-well plate at 1 million cells per well. The number of colonies was counted seven days after plating as previously described 6.

**Induction of differentiation of CD31^+ cells into ECs in vitro**

mBM-CD31^+ cells or hBM-CD31^+ cells were plated onto vitronectin- and fibronectin-coated 2-well chamber slides (1 x 10^5 cells per slide), respectively, and cultured in EGM-2 supplemented with an additional 50 ng/ml VEGF for 8 or 9 days. To evaluate the EC-like phenotype of CD31^+ cells, cells were stained using anti-vWF (Abcam), anti-Flk-1 (BD Biosciences), anti-VE-cadherin (BD Biosciences), or anti-CD31 (BD Biosciences) antibodies. Secondary antibodies, such as FITC-, Alexa 488-, or Alexa 555-conjugated, were appropriately used.

**Transplantation of the CD31^+ and CD31^- cells into ischemic hindlimb**

The hindlimb ischemia was generated as previously described in our group 6. Briefly, a ligation was made around the femoral artery and all arterial branches were removed. One million mBM-
CD31+ or mBM-CD31− cells in 100 µl PBS were intramuscularly injected into the ischemic hindlimb of C57BL/6J mice and the cells in 200 µl PBS were intravenously injected into nude mice with ischemic hindlimb 5, 7. For the human BM cell study, one million hBM-CD31+ cells, hBM-CD31− cells, or hBM-MNCs in 100 µl PBS were intramuscularly injected into the ischemic hindlimb of nude mice 5, 7. In a separate experiment, 1.5 x 10^5 CD34+ cells, CD34−CD31+ cells, and cultured EPCs in 100 µl PBS were injected into the ischemic hindlimb of nude mice, respectively to compare their effects. To compare the vasculogenic effects of hBM-CD34+, -CD34−CD31−, -CD14+, or –CD14−CD31+ cells, 5 x 10^5 cells in 100 µl PBS were injected. Limb necrosis score was calculated by the following rating: 0=none; 1=1–3 tips; 2=4–5 tips; 3=1–3 toes; 4=4–5 toes; 5=1/3 foot; 6=2/3 foot; 8=whole foot; 10=5/3 leg; 12=2/3 leg and 14=whole leg 8.

**Blood flow measurement in ischemic hindlimbs**
Blood flow of the hindlimb was measured using Laser Doppler perfusion imager (LDPI, Moor instrument, UK) as described previously 5. Mean values of perfusion was calculated from the stored digital color-coded images. The level of blood flow of the ischemic (left) limb was normalized to that of non-ischemic (right) limb to avoid data variations caused by ambient light and temperature.

**Measurement of capillary density**
Two weeks and three weeks after the injection of mouse or human cells into ischemic hindlimbs, respectively, the adductor muscles were removed, fixed with 4% paraformaldehyde at 4°C overnight, and frozen-sectioned. To visualize capillaries, the sections were stained using isolectin B4 and DAPI as previously described 1. The stained specimens were visualized under conventional inverted epifluorescence microscopy and the capillary density was calculated from at least 5 randomly selected fields.

**Cell adhesion test**
Ninety-six well plates were pre-coated with 1 µg/well vitronectin (Sigma), 10 µg/well collagen (Sigma, MO), 10 µg/well laminin (BD Biosciences), and 20 µg/well fibronectin (Sigma) respectively at 4°C overnight. Plates were washed with PBS and incubated for 1 hour with 1% bovine serum albumin to block remaining protein binding sites. mBM-CD31+ and mBM-CD31− cells were isolated from GFP-transgenic mice using MACS and 5 x 10^4 cells were transferred to each well and incubated for 12 hr at 37°C in 5% CO2. Unattached cells were removed by gentle washing with PBS and the number of attached cells was determined under fluorescent microscopy.

**Histologic analysis: confocal microscopy**
Before euthanasia, mice were intravenously injected with Alexa 647-conjuaged isolectin B4 as previously described 3. After 10 minutes, mice were euthanized, perfused with normal saline to remove cells and unbound isolectin B4 in the blood vessels, and perfusion-fixed using 4% paraformaldehyde. The hindlimb tissue was removed, fixed in 4% paraformaldehyde at 4°C for 16 hours, and incubated in 30% sucrose solution for 24 hours. Frozen sections of the tissue were obtained at 30 µm thickness. Blood vessels were stained with an EC marker, isolectin B4 (Vector Laboratories) as previously described 1 and nuclei were stained with DAPI (Invitrogen). ECs were identified by positive staining for isolectin B4 and their characteristic morphology within the vascular structure, and transplanted cells were identified by the intrinsic signal of GFP 3, 9. Incorporation of transplanted cells into the vasculature as ECs was determined using a Zeiss LSM 510 Meta confocal laser scanning microscope and LSM 510 Image software (Carl Zeiss, Jena, Germany).
**Real-time RT-PCR**
Total RNA was extracted from cell-injected ischemic hindlimb tissue using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was generated using the TaqMan Multiscribe Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression was determined by TaqMan real-time quantitative PCR (7300 Sequence Detection System, Applied Biosystems) using TaqMan PCR Master Mix (Applied Biosystems). Relative mRNA expression of target gene normalized to GAPDH was calculated by using the formula Relative Expression Level = 2^{-\Delta CT}, where \Delta CT = CT\text{ gene of interest} − \text{CT GAPDH} as previously described\(^1\). The primers and probes were designed using Primer Express 3.0 (Applied Biosystems) and are described in Online Table II.

**Statistical Analysis**
All data were expressed as means ± S.E.M and statistical analysis was conducted using SPSS version 11.0. Student’s t test was used for the statistical analysis for continuous variables between two groups and ANOVA followed by multiple comparison with Bonferroni’s or Tukey’s method for variables among more than 2 groups. The survival analysis was conducted by Kaplan-Meier method. A \(P\) value < 0.05 was considered statistically significant.
Online Figure I. Expression of CD31 in human BM-MNCs
Human BM-MNCs (hBM-MNCs) were isolated and subjected to FACS analysis. Green color represents isotype control. (A) Expression of CD31 in hBM-MNCs. (B) Each bar represents the percentage of CD31+ cells among each cell fraction (n = 3). (C) Expression of hematopoietic lineage markers in hBM-CD31+ cells (n = 3). (D) Each bar represents the percentage of CD31+ cells among each cell type. A large percentage of endothelial cell marker-expressing hBM-
MNCs cells were also positive for CD31 (n = 3). (E) The majority of cells double-positive for hBM-CD31 and endothelial cell markers were also positive for CD45, indicating hematopoietic origin (n = 3).
Online Figure II. Enrichment of hematopoietic stem and progenitor cells in the CD31\(^+\) fraction of human BM

(A) FACS analysis of hBM-MNCs showed that most of the CD34\(^+\) and CD133\(^+\) cells expressed CD31 (n = 3). (B) CD31 expression in CD34\(^+\)CD133\(^+\) cells and CD34\(^+\)CD133\(^-\) cells. All CD34\(^+\)CD133\(^+\) cells and most of the CD34\(^+\)CD133\(^-\) cells expressed CD31 (n = 3).
Online Figure III. Endothelial progenitor cells (EPCs) are found exclusively in the hBM-CD31+ cell fraction in vitro

(A) hBM-CD31+ or -CD31- cells were cultured in complete EGM-2 media. Four days later, cells were incubated with Dil-acLDL and stained with FITC-conjugated BS-1 lectin. Cells double-positive for acLDL and BS-1 lectin were counted as putative EPCs. EPCs were identified almost exclusively in the CD31+ cells, with few in the CD31- cells (**P < 0.01, n = 6 - 7). (B) CD31+ cells were induced to differentiate into ECs by culturing in EGM-2 media supplemented with an additional 50 ng/ml VEGF-A for 8 to 9 days. These cultured CD31+ cells exhibit multiple EC markers, including von Willebrand factor (vWF), Flk-1, CD31, and VE-cadherin (VE-cad). (C)
CFU-Hill assay was performed using hBM-CD31\(^+\) or -CD31\(^-\) cells. The number of CFU-Hill colonies was almost 8 times higher in the CD31\(^+\) cells compared to the CD31\(^-\) cells (**P < 0.001, n = 8).
Online Figure IV. Enhanced repair of hindlimb ischemia by treatment with mBM-CD31+ cells in C57BL/6J mice

One million mBM-CD31+ or -CD31- cells from C57BL/6 mice were intramuscularly injected into the ischemic adductor muscle. (A, B) Blood perfusion of the limb was determined using a laser Doppler perfusion imager (LDPI). The representative LDPI images (A) and quantification of the perfusion (B) showed that hindlimb blood flow was significantly enhanced only in the CD31+ group compared to the PBS-treated control (**P < 0.01, n = 4). (C, D) Two weeks after cell transplantation, hindlimb tissues were harvested and the tissue sections were stained with ILB4 to identify ECs. The CD31+ group, but not the CD31- group, showed significant increase in the number of capillaries as compared to the PBS group (*P < 0.05, n = 5). Bars: C, 100 μm.
Online Figure V. Therapeutic effects of hBM-CD31+ cells on hindlimb ischemia

Three weeks after intramuscular injection of hBM-CD31+ or hBM-CD31− cells into ischemic hindlimbs of athymic mice, the limb salvage (A, B), blood perfusion (C, D), and capillary density (E, F) were determined. Representative photographs of the limbs (A) and limb salvage rate (B) showed higher survival of the limbs in mice treated with CD31+ cells than with CD31− cells or PBS (n = 10). Representative laser Doppler perfusion images (C) and quantitative analysis of the LDPI results (D) demonstrated significantly higher blood perfusion in the CD31+ group than PBS or CD31− groups (vs. CD31+, ##P < 0.01; vs. PBS, **P < 0.01, ***P < 0.001, n = 5). (E, F) The hindlimb tissues were stained with ILB4. Representative photomicrographs (E) and counting of capillaries (F) showed significantly increased capillary density in the CD31+ group compared to the CD31− and PBS groups (*P < 0.05, ***P < 0.001, n = 5). Bars: E, 100 μm.
Online Figure VI. Therapeutic effect of various BM cell populations in an ischemic hindlimb model

After induction of hindlimb ischemia in nude mice, $1.5 \times 10^5$ CD31$^+$ cells, CD34$^-$CD31$^+$ cells, CD34$^+$ cells, and cultured EPCs derived from human BM were intramuscularly injected into ischemic limbs, and limb necrosis (A, B) and limb perfusion (C, D) were determined. Representative photographs of the limbs (A), limb necrosis scores (B), representative laser Doppler perfusion images (C) and quantitative analysis of the LDPI results (D) were shown. There were no differences in the therapeutic effect among these treatment groups ($n = 6 - 12$).
Online Figure VII. The CD31+ cells are more effective in improving hindlimb ischemia than total mononuclear cells

After induction of hindlimb ischemia in nude mice, $1 \times 10^6$ CD31+ cells, high number ($2.5 \times 10^6$ cells) of total mononuclear cells including approximately $1 \times 10^6$ CD31+ cells (high number MNCs), or low number ($1 \times 10^6$) total mononuclear cells (MNCs) were injected into ischemic limbs and the limb necrosis (A, B) and limb perfusion (C, D) were determined. Representative photographs of the limbs (A) and limb necrosis scores (B) are shown. Limb necrosis in the CD31+ cell group was significantly lower than high number MNC injected groups (**$P < 0.01$, $n = 8$). Representative laser Doppler perfusion images (C) and quantitative analysis of the LDPI results (D) are shown. Hindlimb perfusion was significantly higher in the CD31+ cell group than high number or low number- MNC groups (**$P < 0.01$, *$P < 0.05$, $n = 8$).
Online Figure VIII. The therapeutic effects of Lin$^{CD31^+}$ cells and Lin$^{CD31^-}$ cells are comparable in improving hindlimb ischemia

After induction of hindlimb ischemia in nude mice, $5 \times 10^5$ mouse BM-Lin$^{CD31^+}$ or -Lin$^{CD31^-}$ cells were injected into the ischemic limbs and limb necrosis (A, B) and blood perfusion (C, D) were determined. Representative photographs of the limbs (A), limb necrosis scores (B), representative LDPIs (C) and quantitative analysis of the LDPI results (D) showed no difference in the therapeutic effects between the Lin$^{CD31^+}$ and Lin$^{CD31^-}$ cell groups ($n = 7 - 9$).
Online Figure IX. Therapeutic effects of CD31⁺ and CD31⁻ cells isolated from ApoE-KO mice in hindlimb ischemia.

After induction of hindlimb ischemia in nude mice, 1 x 10⁶ cells of the BM-CD31⁺ cells or -CD31⁻ cells from ApoE KO mice, or BM-CD31⁺ cells from normal C57BL/6J mice were injected into ischemic limbs and the limb necrosis (A, B) and limb perfusion (C, D) were determined. Representative photographs of the limbs (A) and limb necrosis scores (B) show that limb necrosis in the ApoE KO CD31⁺ cell group was significantly lower than the ApoE KO CD31⁻ cell group (*P < 0.05, n = 9 - 10) and that there was no difference in limb necrosis rate between ApoE KO CD31⁺ cell and normal CD31⁺ cell groups, suggesting that ApoE KO CD31⁺ cells are as therapeutically effective as normal CD31⁺ cells. Representative LDPIs (C) and quantitative analysis of the LDPI results (D) demonstrated that there was no statistically significant difference in blood perfusion between ApoE KO CD31⁺ and normal CD31⁺ cell groups (n=9-10). However, blood perfusion was significantly lower in the ApoE KO CD31⁻ cell group compared to the normal CD31⁺ cell group (*P < 0.05, n = 9 - 10).
Online Figure X. Comparison of the effects of CD34+ and CD34−CD31+ cells on neovascularization

Two weeks after injection of 1.5 x 10^5 hBM-CD34+ or hBM-CD34−CD31+ cells into ischemic hindlimbs of athymic mice, the mice were euthanized and hindlimb tissues were harvested and stained with ILB4 to identify blood vessels. Representative photomicrographs (A) and counting of capillaries (B) showed that there was no difference in capillary density between the two groups. n = 7. Bars: 100 μm.
Online Figure XI. Confocal images of the engrafted cells
The CD31+ and CD31- cells were isolated from GFP transgenic mice and intramuscularly injected into ischemic hindlimbs. Two weeks after transplantation, the engrafted cells in the ischemic hindlimbs were observed under confocal microscopy. Bars: 50 μm.
Online Figure XII. Representative confocal images of vasculogenesis by CD31+ cells in the ischemic tissue

mBM-CD31+ cells from C57BL6/J mice were labeled with Dil (red) and intramuscularly injected into ischemic hindlimbs. One week after the transplantation, mice were euthanized and the ischemic limbs were removed. The tissue was stained using ILB4 (green) and DAPI (blue) to visualize the blood vessels and nuclei, respectively. mBM-CD31+ cells incorporated into vascular structures and expressed ILB4, suggestive of vasculogenesis. Bars: 20 µm.
Online Figure XIII. Vasculogenic effect of various populations of human bone marrow (hBM) cells in the ischemic tissue

hBM-CD34+ or -CD34-CD31+ cells (A, B) or hBM-CD14+ or -CD14-CD31+ cells (C, D) were labeled with Dil and intramuscularly injected into ischemic hindlimbs of athymic mice. Two weeks after cell transplantation, Alexa 647-conjugated ILB4 was injected into tail vein to stain functional endothelial cells (ECs). The hindlimb tissues were enzymatically digested and subjected to flow cytometry to determine ECs derived from the transplanted cells. (A, B) Representative flow cytometric analyses in each experiment. (A, B) There was no difference in the number of ECs derived from the transplanted cells between the CD34+ and the CD34-CD31+ cells (n = 6). (C, D) The number of ECs derived from injected was significantly higher in the CD14-CD31+ cell-injected group than in the CD14+ cell-injected group (*P < 0.05, n = 5 - 6).
### Pro-angiogenic gene set

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<tr>
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### Anti-angiogenic gene set

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### Online Table II. Sequences of primers and probes for real-time RT-PCR.

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Supplemental References


Legend for Video 1. Representative projection view of the engrafted CD31\(^+\) cells.
The CD31\(^+\) cells were isolated from GFP transgenic mice and intramuscularly injected into ischemic hindlimbs. Two weeks after transplantation, the engrafted cells in the ischemic hindlimbs were observed under confocal microscopy.

Legend for Video 2. Representative projection view of the engrafted CD31\(^-\) cells.
The CD31\(^-\) cells were isolated from GFP transgenic mice and intramuscularly injected into ischemic hindlimbs. Two weeks after transplantation, the engrafted cells in the ischemic hindlimbs were observed under confocal microscopy.

Legend for Video 3. Representative projection view of the confocal images of Figure 8B.
mBM-CD31\(^+\) cells were isolated from GFP-transgenic mice and intramuscularly transplanted into ischemic hindlimbs. One week after transplantation, Alexa 647-conjugated ILB4 was intravenously injected into mice to stain functional endothelial cells. Blue, DAPI (nuclei); green, GFP (transplanted CD31\(^+\) cells); red, \textit{in vivo} ILB4 perfusion staining. Three-dimensional reconstruction of z-stacked confocal microscopic imaging demonstrated that GFP-positive mBM-CD31\(^+\) cells were colocalized with functional (\textit{in vivo} ILB4-positive) endothelial cells, suggesting vasculogenesis from the transplanted CD31\(^+\) cells.