addition of streptavidin-conjugated magnetic beads and BD IMagnet separation (Takahashi et al., 1999; Otani et al., 2002; Rafii and Lyden, 2003). Then, Lin- MNCs were counted and the number of Sca1+Lin− cells was calculated from the rate of Sca1+ cells in the Lin− MNCs by FACS analysis and the number of Lin− MNCs.

Regular flow cytometric profiles were analyzed with a FACS Calibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA) in a previously described procedure (Iwasaki et al., 2006; Matsumoto et al., 2006). The following monoclonal antibodies were used to characterize the lineage-depleted MNCs: APC-conjugated anti-cKit (BD Pharmingen), FITC conjugated anti-Sca1 (BD Pharmingen), IgG1-PE isotype controls (BD Pharmingen), IgG1-FITC isotype controls (BD Pharmingen), PI (Sigma Co).

Mouse BMT model and detection of β-galactosidase/Tie-2 expression at the fracture site

This procedure was performed as previously described (Asahara et al., 1999; Takahashi et al., 1999). FVB/N mice underwent BMT from transgenic mice constitutively expressing β-galactosidase encoded by lacZ under the transcriptional regulation of an EC-specific promoter, Tie-2. Briefly, BM cells were obtained by flushing the femurs and tibias of age-matched (10-week-old), donor Tie-2 transgenic mice. Low-density BM MNCs were isolated by density gradient centrifugation with Histpaque-1083 (Sigma). Approximately 2×10^6 donor BM MNCs were intravenously transplanted into FVB/N mice lethally irradiated with 12.0 Gy. At 4 weeks after transplantation, by which time the BM of the recipient mice was reconstituted with the donor cells, the Tie2/LZ/BMT mice underwent surgery for fracture creation. Restricted expression of LacZ was confirmed only in the reconstituted BM, not in other somatic cells before creating fractures. Mice were killed 1 and 4 weeks post-fracture for histological assessment. Mouse femurs in OCT blocks were sectioned, and 6 μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 min and incubated in X-gal solution overnight at 37°C. Histological sections were counterstained with light hematoxylin and eosin and examined by light microscopy. The control samples from contralateral limbs of BMT mice were examined identically (n = 5 in each group).

Isolation of EPC-enriched fraction from PB for transplantation study

BMT was performed to detect the BM-derived EPCs mobilized and recruited into the fracture site, however, EPCs directly migrating from the fractured BM to the damaged tissue might also be detected by this assay. Therefore, we performed an additional study to confirm the recruitment of circulating EPCs through the vascular route.

PB-MNCs were isolated from fractured GFP mice as described above. Sca1+ and Lin− fraction and Sca1−Lin+ cells of the PB-MNCs (Sca1+Lin− cells, Sca1−Lin+ cells) were sorted by FACSAria™ (Becton Dickinson Immunocytometry Systems) (Sato et al., 1999; Sahara et al., 2005). Prior to the sorting procedure, PB-MNCs were stained with the above described cocktail of biotinylated monoclonal antibodies against lineage markers for 20 min at 4°C. The cells were stained with a PE-conjugated anti-Sca1 antibody (BD Pharmingen) and APC-Cy7-conjugated streptavidin (BD Pharmingen) for 20 min at 4°C.

To target cells transplanted intravenously and confirm their recruitment into the peri-fracture site, 5×10^4 PB Sca1+Lin− or Sca1−Lin+ cells from fractured GFP mice resuspended with 50 μl of PBS were intravenously transplanted into three additional fractured mice through their tail veins (n = 5 in each group). To detect injected PB Sca1+Lin− cells (GFP+ cells)-derived EC differentiation at the fracture site, all animals were, following the radiological assessment, killed at weeks 1 and 4 and double immunohistochemistry was performed with rat anti-mouse PECAM-1 (CD31) and goat anti-mouse GFP (Molecular Probes) antibodies. The secondary antibodies for each immunostaining are as follows: FITC-conjugated anti-rat IgG (H + L) (Jackson) for PECAM-1 and Cy3-conjugated goat anti-goat IgG (H + L) (Jackson) for GFP staining. DAPI solution was applied for 5 min for nuclear staining.

Statistical analysis

All values were expressed as mean ± SE. Paired *t* tests were performed for comparison of data before and after fracture. The multiple comparisons among groups were made using the one-way analysis of variance (ANOVA). Post hoc analysis was performed by Fisher's PLSD test. The analyses were performed using a statistical software package (Statview 5.0, Abacus Concepts, Inc., Berkeley, CA). A probability value <0.05 was considered to denote statistical significance.

Results

Neoangiogenesis and blood flow recovery in the early phase of fracture healing

Morphological fracture healing was evaluated by radiological examinations. In 35% (7 of 20) at week 2, 90% (18 of 20) at week 3, and all (20 of 20) animals at week 4, the fracture radiographically healed with bridging callus formation (Fig. 1A). In all animals (20 of 20) at week 4, callus absorption was found as a general sign of chronic stage of bone healing. These findings were consistent with a previous report demonstrating the natural course of this animal model (Manigrasso and O'Connor, 2004).

Morphological fracture healing was also evaluated by histological examinations. Histological evaluation with toluidine blue staining demonstrated the general time course as follows: no granulation at week 0, a callus formation at week 1, enhanced endochondral ossification consisting of numerous number of

Fig. 1. Neoangiogenesis and blood flow recovery in the early phase of fracture healing process. A: Serial X-ray findings taken from a mouse demonstrating representative bone healing after fracture. White arrows indicate the fracture sites and red arrows show bridging callus formation, defined as union. B: Representative and serial histological findings of fracture healing. Toluidine blue staining demonstrates a callus formation at week 1, enhanced endochondral ossification consisting of numerous numbers of chondrocytes and newly formed trabecular bone at week 2, bridging callus formation at week 3, and complete union and bone remodeling at week 4. C: Serial vascular staining with isolectin B4 (marker for mouse EC) post-fracture (×200). Neovascularization was enhanced at week 1 around the endochondral ossification area. Green fluorescence shows isolectin B4 for capillary staining and blue fluorescence indicates DAPI for nuclear staining. D: Representative laser Doppler perfusion imagings (LDPIs) immediately after (week 0), 1, 2, 3, and 4 weeks following fracture. In these digital color-coded images, maximum perfusion values are indicated in white, medium values are in yellow, and lowest values are in dark blue. The skin blood flow within the fracture site (red square) and intact contralateral site (black square) are calculated by LDPI. LDPI analysis demonstrated a severe reduction of blood flow at the fracture sites compared to contralateral sites 1 h after fracture creation and serial recovery thereafter. E: Serial changes in histological capillary density at the fracture sites. Neovascularization was significantly enhanced at weeks 1 and 2 compared to other time points. n = 5 for each time point. ††$P < 0.01$ for week 1 versus weeks 0, 3, 4. †$P < 0.05$ for week 2 versus weeks 0, 3, 4. F: Serial changes in the flux ratio of fractured/intact (contralateral) limb following fracture. The flux ratio peaked at week 1 and significantly increased at weeks 1 and 2 compared to other time points. n = 5 for each time point. ††$P < 0.01$ for week 1 versus weeks 0, 3, 4. †$P < 0.05$ for week 2 versus weeks 0, 3, 4.

chondrocytes and newly formed trabecular bones at week 2, bridging callus formation at week 3, and complete union (and bone remodeling) at week 4 (Fig. 1B).

Enhanced angiogenesis during the fracture healing were confirmed by immunostaining. Serial vascular staining with isolectin B4 (marker for mouse EC) post-fracture demonstrated enhanced neovascularization at week 1 around the endochondral ossification area (Fig. 1C). Neovascularization assessed by capillary density was significantly enhanced at week 1 compared to other time points

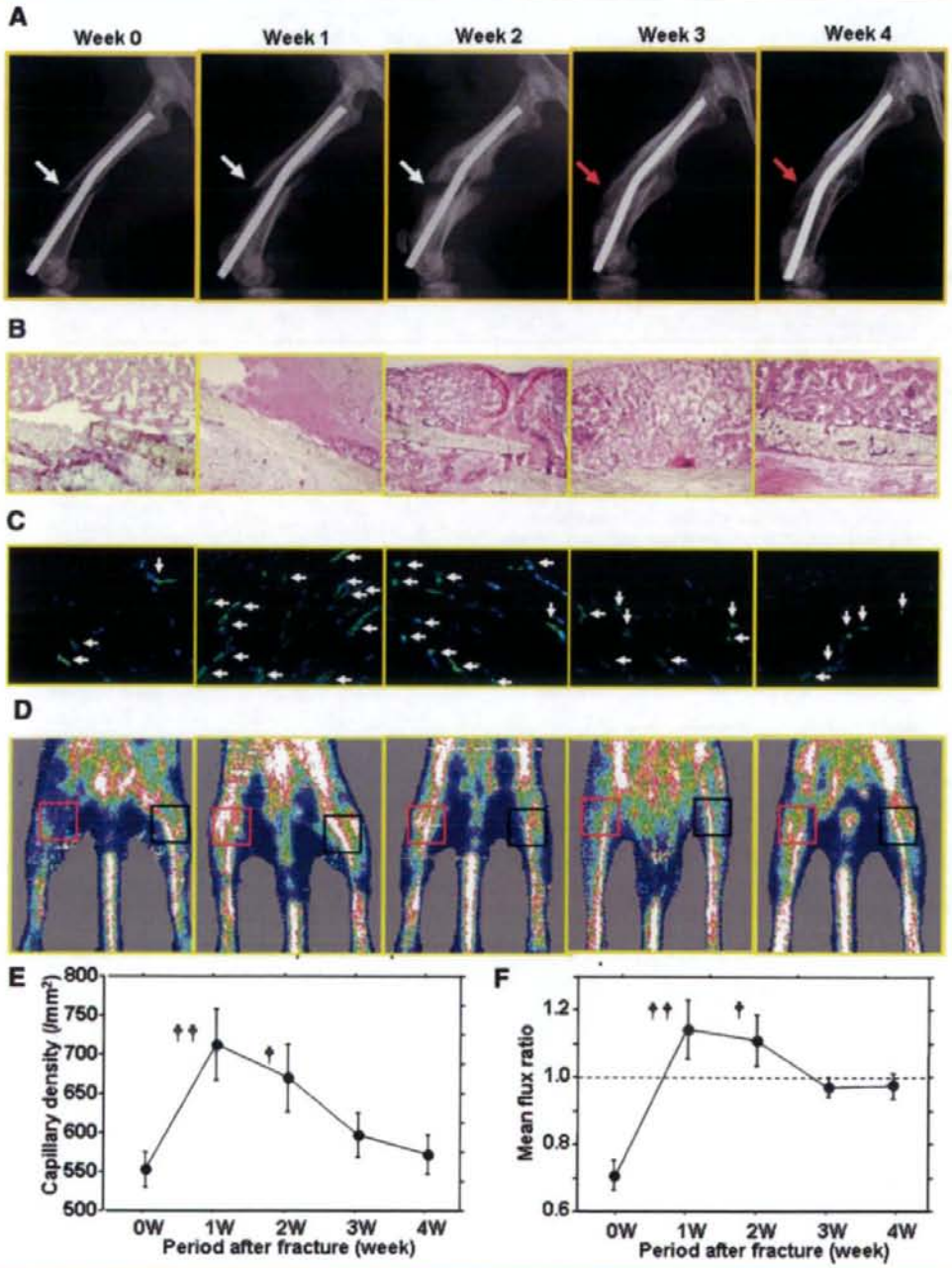

Fig. 1.

(week 0, 557.5 ± 22.4/mm^2; week 1, 712.5 ± 45.4/mm^2; week 2, 670.0 ± 43.1/mm^2; week 3, 597.5 ± 28.0/mm^2; week 4, 572.5 ± 25.1/mm^2, respectively. $P < 0.01$ for week 1 vs. weeks 0, 3, and 4, $P < 0.05$ for week 2 vs. weeks 0, 3, and 4) (Fig. 1E).

LDPI was serially performed after fracture to evaluate blood flow recovery at the fracture site as a physiological approach. LDPI analysis demonstrated a severe reduction of blood flow at the fracture sites 1 h after fracture creation (week 0) and its following recovery (Fig. 1D). In proportion to the serial change of capillary density, the ratio of fractured/intact (contralateral) blood flow significantly increased at week 1 compared to other time points (week 0, 0.71 ± 0.05/mm^2; week 1, 1.14 ± 0.09/mm^2; week 2, 1.11 ± 0.08/mm^2; week 3, 0.98 ± 0.03/mm^2; week 4, 1.02 ± 0.06/mm^2, respectively. $P < 0.01$ for week 1 vs. weeks 0, 3, and 4, $P < 0.05$ for week 2 vs. weeks 0, 3, and 4) (Fig. 1F).

These results indicate that histological neoangiogenesis activity may correlate well with functional blood flow recovery at the fracture site and that these phenomena may occur at an early phase of fracture healing in advance of endochondral ossification in later phase.

Phenotypic characterization of BM and PB pre and post-fracture

We first attempted to compare the frequency of bone marrow HSC/EPC fraction identified as cKit+Sca1+Lin− (KSL) cells between pre and 7 day post-fracture in 10-week-old male mice. The BM contained a KSL fraction at a significantly higher post-fracture rate compared to pre-fracture (pre-fracture, 5.6 ± 0.2%; post-fracture, 8.4 ± 0.5%, respectively, $P < 0.05$, $n = 5$) (Fig. 2A), as determined by FACS analysis. Number of KSL cells in 10^5 MNCs was significantly greater post-fracture compared to pre-fracture (pre-fracture, 581.9 ± 97.9; post-fracture, 1,212.0 ± 253.0, respectively, $P < 0.05$) (Fig. 2B).

We next investigated which cell populations were mobilized into PB under fracture stress. Compared to BM cells, the PB contained little number of KSL cells and there was no significant difference between the number and percentage of pre and 7-day post-fracture (data not shown). Following this, we attempted a comparison of the frequency of HSC/EPC-enriched fraction identified as Sca1+ Lin− cells between pre and 7 day post-fracture in the 10-week-old male mice. The BM contained the HSC/EPC-enriched fraction at a significantly higher rate post-fracture than pre-fracture (pre-fracture, 9.2 ± 0.6%; post-fracture, 14.2 ± 1.0%, respectively, $P < 0.05$) (Fig. 2C), as determined by FACS analysis. PB also contained the HSC/EPC-enriched fraction at a significantly higher rate post-fracture than pre-fracture (pre-fracture, 44.9 ± 0.8%; post-fracture, 55.0 ± 1.2%, respectively, $P < 0.05$) (Fig. 2C), as determined by FACS analysis. The number of Sca1+Lin− cells per 10^5 MNCs of BM was greater post-fracture compared to pre-fracture (pre-fracture, 955.6 ± 143.2 cells/10^5 MNCs, post-fracture, 2,022.3 ± 370.3 cells/10^5 MNCs, respectively, $P < 0.05$) (Fig. 2D). The number of Sca1+Lin− cells per 1 ml of PB was also larger post-fracture compared to pre-fracture (pre-fracture, 7.2 ± 1.1 × 10^4 cells/ml, post-fracture, 13.7 ± 1.9 × 10^4 cells/ml, respectively, $P < 0.05$) (Fig. 2D).

These results indicate that fracture-stress may increase the number of the HSC/EPC population in BM and induce mobilization of the BM stem/progenitor cells into PB post-fracture.

BM Sca1+ Lin− cell-derived vasculogenesis

Based on the results that PB Sca1+ Lin− cells increased following fracture, we performed experiments to identify BM-derived EPC incorporation around the fracture sites. First, to histologically prove the phenomenon of Sca1+ cell-derived vasculogenesis, double immunohistochemical staining for Sca1, a stem cell marker, and CD31, an EC marker, was performed using tissue samples obtained 1 and 4 weeks after fracture. Sca1+ cell-derived mature ECs were more abundantly detected as the double positive cells for CD31 and Sca1 in vasculature of the peri-fracture area, while the double positive cells were rarely identified in non-fracture area at week 1 (Fig. 3A). Even after complete union at week 4, Sca1+ cell-derived mature ECs were also abundantly detected in vasculature of the peri-fracture area, while few double positive cells were identified in non-fracture area (Fig. 3A). The number of the double positive cells was larger in the peri-fracture area compared to non-fracture area at both 1 and 4 weeks after fracture (week 1: fracture site, 280.0 ± 20.0/mm^2; non-fracture site, 30.0.0 ± 12.2/mm^2, week 4: fracture site, 165.0 ± 12.2/mm^2; non-fracture site, 25 ± 13.4/mm^2, respectively. $P < 0.01$ for week 1 and 4) (Fig. 3B). Whole vascularization assessed by CD31-positive capillary density was also significantly enhanced in the peri-fracture area compared to non-fracture area at weeks 1 and 4 (week 1: fracture site, 625.0 ± 54.2/mm^2; non-fracture site, 415.0 ± 34.1/mm^2, week 4: fracture site, 500.0 ± 53.7/mm^2; non-fracture site, 385.0 ± 41.5/mm^2, respectively. $P < 0.01$ for weeks 1 and 4) (Fig. 3B).

Furthermore, we used BMT model to obtain direct evidence of enhanced BM-derived EPC incorporation into foci of neoveasularization at the fracture site. Tissue samples 1 week post-fracture demonstrated a significant increase in cells expressing β-galactosidase/Tie-2 in the fracture sites compared with those in the control contralateral limb (Fig. 3C). Even after complete union at week 4, cells expressing β-galactosidase/Tie-2 were detected in the fracture sites, while no β-galactosidase/Tie-2-positive cells were identified in non-fracture area (Fig. 3C).

To support our hypothesis regarding recruitment of BM-derived EPCs into peri-fracture site through vascular route (not by direct migration from fractured BM), PB Sca1+Lin− or Sca1−Lin+ cells from GFP-transgenic mice were intravenously transplanted into fractured wild-type mice (Fig. 4A). Immunohistochemistry with tissue samples 1 and 4 weeks post-fracture revealed existence of the double positive cells for GFP and CD31 at the fracture site in mice receiving Sca1+Lin− cells, but not in Sca1−Lin+ group (Fig. 4B). Whole vascularization assessed by CD31-positive capillary density was significantly enhanced in animals receiving Sca1+Lin− cells compared with Sca1−Lin+ group at weeks 1 and 4 (week 1: Sca1+Lin−, 730.4 ± 32.7/mm^2; Sca1−Lin+, 610.7 ± 28.7/mm^2, week 4: Sca1+Lin−, 614.3 ± 22.5/mm^2; Sca1−Lin+, 505.4 ± 25.8/mm^2, respectively. $P < 0.01$ for weeks 1 and 4) (Fig. 4C).

These results strongly indicate that BM-derived EPCs may be incorporated into the fracture site through vascular route for vasculogenesis in the fracture healing process.

Discussion

The serial stages of fracture healing are well understood as cellular processes. Following the initial haematoma formation, a cartilaginous callus bridges the fracture gap, while intramembranous new bone forms the buttress of the bridge (Kernek and Wray, 1973; Brighton and Hunt, 1997). In this process, neovascularization is recognized as a crucial initiator of bone formation and remodeling. However, it is still unclear which type of cells contributes to the development of endochondral field and how the neovascularization process originates and is enhanced. Vasculogenesis by EPCs, which is involved in the development of the blood vessel system in the embryonic stage (Risau et al., 1988; Pardanaud et al., 1989), had

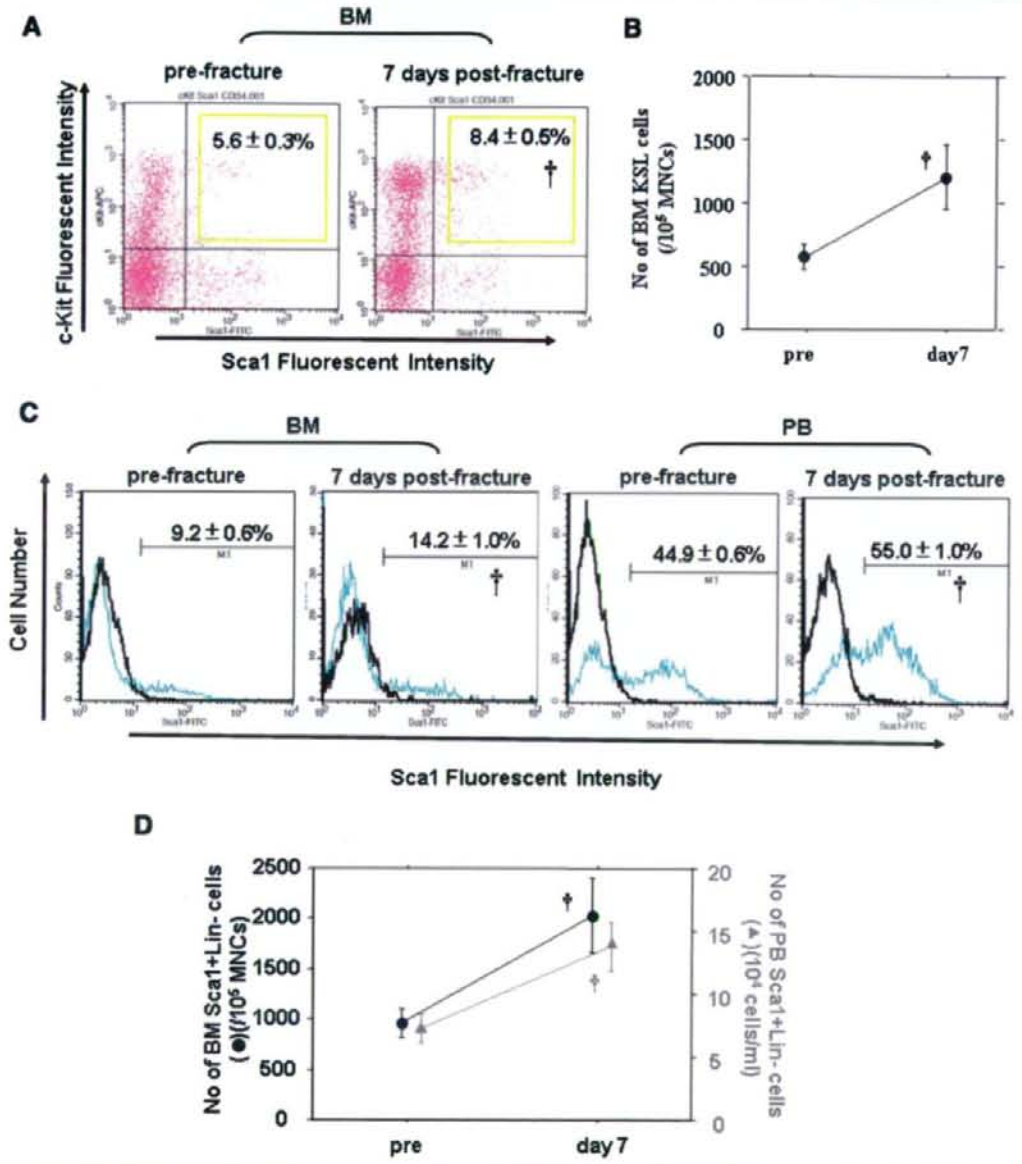

Fig. 2. Phenotypic characterization of bone marrow (BM) and peripheral blood (PB) cells pre and post-fracture. A: BM cells of pre- and 7 days post-fractured mice assessed by FACS analysis. Fracture-stress significantly increased the percentage of cKit+Sca1+ cells in Lineage− (Lin−) mononulear cells (MNCs) in BM. †$P < 0.05$ for pre versus day 7. B: The number of cKit+Sca1+Lin− (KSL) cells in 10^5 BM MNCs was significantly greater post-fracture compared to pre-fracture. n = 5 for each time point. †$P < 0.05$ for pre versus day 7. C: BM and PB cells of pre- and 7-day post-fractured mice assessed by FACS analysis. Percentage of Sca1+ cells in Lin-MNCs significantly increased following fracture in BM and PB. †$P < 0.05$ for pre versus day 7. D: The number of Sca1+Lin− cells in BM and PB. The number of Sca1+Lin− cells in BM and PB was significantly greater post-fracture than pre-fracture. n = 5 for each time point. †$P < 0.05$ for pre versus day 7. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not been identified as a mechanism of post-natal endothelial regeneration until our discovery of BM-derived and circulating EPCs in adults (Asahara et al., 1997, 1999; Takahashi et al., 1999). Then, we and many researchers have applied the EPCs for therapeutic neovascularization and acquired beneficial results (Kalka et al., 2000; Murohara et al., 2000; Kawamoto et al., 2001, 2003; Kocher et al., 2001; Assmus et al., 2002; Britten et al., 2003; Sivan-Loukianova et al., 2003; Werner et al.,

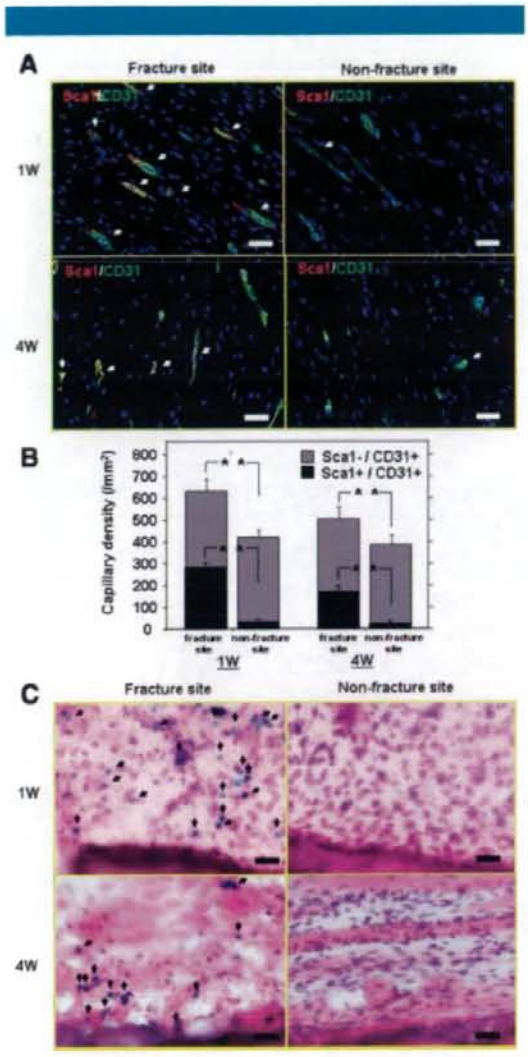

Fig. 3. BM Sca1+Lin− cell-derived vasculogenesis. **A: Immunohistochemical staining for Sca1 (red) and CD31 (green) using tissue samples 1 and 4 weeks post-fracture. Abundant distribution at week 4 as well as week 1 of Sca1+ cell-derived ECs (arrow) were identified at the peri-fracture site, whereas the double positive cells were rarely identified at the non-fractured site. Blue fluorescence indicates DAPI for nuclear staining. Scale bars: 20 μm. B: Whole and Sca1+ cell-derived vascularization assessed by CD31-positive and CD31+/Sca1+ capillary density was significantly enhanced in the peri-fracture area compared to non-fracture area at weeks 1 and 4. n = 5 in each group. $^{**}P < 0.01$. C: Chemical staining for β-galactosidase using tissue samples 1 and 4 weeks post-fracture of animal model of BM transplantation (BMT) from transgenic donors expressing LacZ transcriptionally regulated by endothelial cell-specific Tie-2 promoter. Cells expressing Tie2/β-galactosidase (arrows) were abundantly identified at week 1 and detected even at week 4 at the fracture site of mice undergoing BMT. In contrast, the Tie2/β-galactosidase expressing cells were not identified at the control non-fractured site. Scale bars: 20 μm.**

2003; Taguchi et al., 2004; Iwasaki et al., 2006). In the immunodificient rat model of acute myocardial infarction, transplanted human CD34+ cells or ex-vivo expanded EPCs incorporate into the site of the myocardial neovascularization, differentiate into mature ECs, augment capillary density, inhibit myocardial fibrosis and apoptosis, and preserve the left ventricular function (Kawamoto et al., 2001, 2003; Kocher et al., 2001). Systemic administration of human cord blood-derived CD34+ cells to immunocompromised mice subjected to stroke 48 h earlier induces neovascularization in the ischemic zone and provides a favorable environment for neuronal regeneration (Taguchi et al., 2004). Transplantation of peripheral blood CD34+ cells to promote revascularization improves wound healing in full-thickness skin wounds of diabetic mice (Sivan-Loukianova et al., 2003). Following these promising reports, we quite recently reported that human CD34+ cell transplantation induced significant vasculogenesis in regenerating tissues and enhanced functional recovery from non-healing fractures in small animal models (Matsumoto et al., 2006). However, pathophysiological role of EPCs in fracture healing was still unclear. Therefore, we investigated the kinetics of EPCs in BM, PB, and fracture sites during the healing process.

In the present study, we utilized a reproducible animal model of femur fracture with severe decrease in local blood flow, physiologically proven by LDPI examination. The natural history of this model is clearly relevant to the clinical situation of the common fracture. In this model, serial change in local blood flow was parallel to that in capillary density, which peaked at day 7. At the peak time of this blood flow recovery, significant increase of BM KSL cells and BM and PB Sca1+Lin− cells, which are EPC-enriched fractions, was also confirmed by FACS analysis. Eghbali-Fatourechi et al. (2005, 2007) and Khosla and Eghbali-Fatourechi (2006) reported that human osteoblast-lineage cells co-expressing CD34 were increased in PB of patients with bone fracture. Taken together with these clinical reports, the present results indicate that BM EPCs may be mobilized into circulation in the acute phase of fracture healing.

In addition, the mobilized Sca1+Lin− cells were immunohistochemically proved to incorporate into the fracture site and differentiate into mature ECs. Recently, Ford demonstrated that CD34+ cells line the cavities of the cartilage in the fracture site in a rabbit tibial osteotomy model (Ford et al., 2004). In our previous study, transplanted human CD34+ cells were shown to differentiate into ECs at the fracture site of an immunodeficient rat fracture model (Matsumoto et al., 2006). In this study, a mouse model of BM transplantation from transgenic donors expressing LacZ transcriptionally regulated by endothelial cell-specific Tie-2 promoter first provided direct evidence that EPCs contributing to neovascularization at the fracture site were specifically derived from BM. However, it was still unclear whether recruitment of the BM-derived EPCs is due to direct migration from the fractured BM or the homing of circulating cells through vascular routes. Therefore, we performed a further study to clarify this by double immunostaining for GFP and CD31 with tissue samples 1 and 4 weeks post-fracture and intravenous transplantation of GFP+/Sca1+/Lin− cells. In this assay, abundant distribution of the double positive cells at the fracture site was observed following PB GFP+/Sca1+/Lin− cell transplantation. These series of studies strongly suggest that circulating EPCs mobilized from BM may home to the fracture site and play an important role in fracture healing via functional EC differentiation. As we expected, this EPC kinetics for the fracture healing was consistent with the previous findings in ischemic disease, cancer and wound healing (Asahara et al., 1999; Takahashi et al., 1999). However, our histological study also demonstrated that part of neovascularization at the fracture site is independent of vasculogenesis by BM-derived

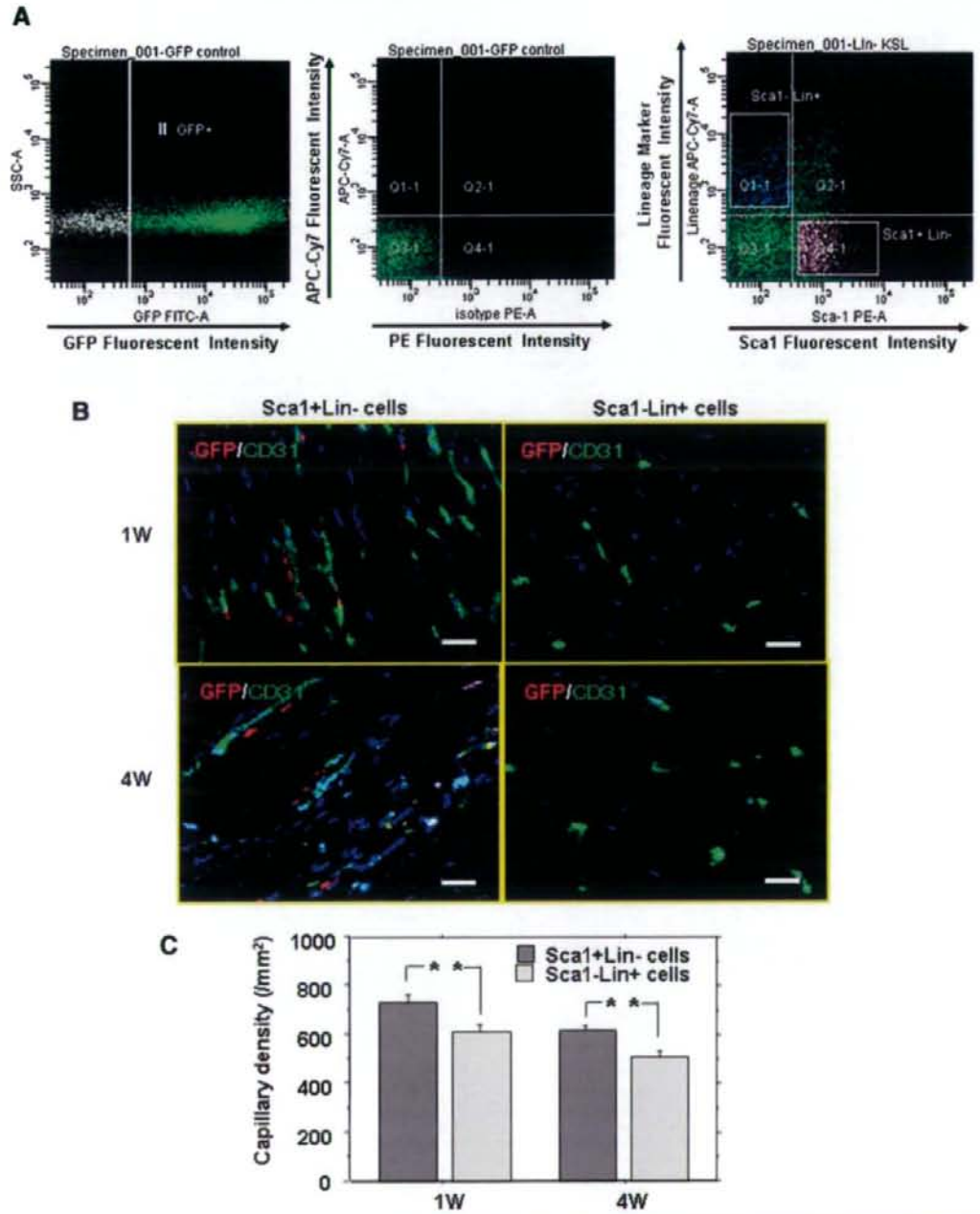

Fig. 4. Contribution of PB Sca1+Lin− cells to fracture healing. A: Representative example of FACSAria sorting of PB-GFP+/Sca1+/Lin− or GFP+/Sca1−/Lin+ cells from fractured GFP mice. Green color population indicated GFP+ cells in PB of GFP transgenic mice (Left part). Middle part shows PE- and APC-Cy7-isotype control. After GFP+ cell selection, PB-Sca1+/Lin− cell (Q4-1: white) or Sca1−Lin+ cell population (Q1-1: blue) were sorted (Right part). B: Double immunostaining for GFP (red) and CD31 (green) with tissue samples 1 and 4 weeks post-fracture and intravenous transplantation of GFP+/Sca1+/Lin− or GFP+/Sca1−/Lin+ cells. More abundant distribution of the double positive cells were identified at the fracture site following PB GFP+/Sca1+/Lin− cell transplantation compared with Sca1−/Lin+ cell infused group. Blue fluorescence indicates DAPI for nuclear staining. Scale bars: 20 μm. C: Whole vascularization assessed by CD31-positive capillary density was significantly enhanced in animals receiving Sca1+Lin− cells compared with Sca1−Lin+ group at weeks 1 and 4. n = 5 in each group. $^{**}P < 0.01$.

EPCs, suggesting other mechanisms such as paracrine effect of the BM-derived EPCs on resident EPCs and ECs, vasculogenesis by local EPCs or angiogenesis by the resident ECs. Our previous study demonstrated that transplanted CD34+ cells secreted angiogenic factors including VEGF, FGF2, and HGF may be also involved in bone healing at the fracture site, and that the inhibition of angiogenesis by soluble Flt1 (VEGF antagonist) suppressed not only angiogenesis/vasculogenesis but also intrinsic osteogenesis, indicating that angiogenic factors released by the transplanted CD34+ cells, at least in part, contribute to fracture healing in paracrine manner (Matsumoto et al., 2006).

The endochondral ossification and callus formation was advanced following the early phase of fracture healing process, suggesting that the initial stage of neovascularization by EPCs is considered to be crucial for complete fracture healing in the late phase. These pathophysiological findings suggest the demand of EPCs at the fracture sites and the therapeutic usefulness in future clinical application. In conclusion, neovascularization in the early phase of fracture healing is modulated by mobilization of BM-derived EPCs into circulation and their incorporation into fracture site, leading to a contribution to bone healing.

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Endothelial Progenitor Cells for Cardiovascular Regeneration

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Abstract

Endothelial progenitor cells (EPCs) are peripheral blood mononuclear cells that can differentiate into mature endothelial cells. Adult EPCs were first discovered in human peripheral blood in 1997. Since then, the potency of EPCs for cardiovascular regeneration has been demonstrated in several preclinical studies, and investigators are beginning to evaluate the therapeutic utility of EPCs in early-phase clinical trials. This review summarizes the progression of basic, preclinical, and clinical research into the potential use of EPC therapy for cardiovascular regeneration.

Introduction

Endothelial progenitor cells (EPCs) are immature cells capable of differentiating into mature endothelial cells. In 1997, EPCs were first identified in adult human peripheral blood as CD34 antigen-positive (CD34+) mononuclear cells (MNCs) (Asahara et al. 1997). Since this discovery, the pathophysiological role and therapeutic application of adult (i.e., postnatal) EPCs have been the subjects of intense experimental and clinical investigation. This review summarizes the progress of basic, preclinical, and clinical research into this novel therapeutic modality.

Characteristics of Postnatal EPCs

Endothelial progenitor cells and hematopoietic stem cells (HSCs) share many surface marker antigens, such as Flk-1, Tie-2, c-Kit, Sca-1, AC133, and CD34 (Flamme and Risau 1992, His 1900, Weiss and Orkin 1996), and likely descend from a common precursor cell (hemangioblasts) during embryonic development. In 1997, Asahara et al. isolated CD34+ MNCs from human peripheral blood, cultured them on fibronectin, then tested them for the expression of leukocyte (CD45) and endothelial cell (CD34, CD31, Flk-1, Tie-2, E selectin, and endothelial nitric oxide synthase [eNOS]) markers to confirm that the cultured cells developed an EC-like phenotype. After intravenous injection into immunodeficient mice with experimental hindlimb ischemia, fluorescent dye-labeled CD34+ cells were incorporated into the sites of ischemic neovascularization. On the basis of this compelling evidence, the isolated

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CD34+ MNCs were identified as postnatal EPCs. Since the publication of this initial report, several groups have used a variety of methodologies to identify EPCs (Gehling et al. 2000, Gunsilius et al. 2000, Lin et al. 2000, Peichev et al. 2000, Shi et al. 1998).

Like HSCs, adult EPCs are believed to originate from the bone marrow; thus, transplantation of bone marrow containing labeled EPCs enables cells descended from the bone marrow to be identified after incorporation into various tissues. Bone marrow-derived EPCs have been found in foci of neovascularization during wound healing and tumor growth, in ischemic skeletal and cardiac muscle, and in corneas after corneal micropocket surgery. Similar EPC incorporation has been observed during endometrial neovascularization after induced ovulation or estrogen administration (Asahara et al. 1999a). These findings suggest that the bone marrow is a major source of EPCs and that endogenous mobilization of EPCs from the bone marrow into the peripheral blood occurs in response to a physiological need for neovascularization. The mobilized EPCs are then recruited into the foci of neovascularization and contribute to new blood vessel formation (Figure 1). This mechanism is also supported by clinical observations of EPC mobilization in patients with burns (Gill et al. 2001) or acute myocardial infarction (Shintani et al. 2001) and in patients undergoing coronary artery bypass graft surgery.

Therapeutic Modulation of EPCs

Techniques that influence EPC mobilization and incorporation could enhance EPC therapy both directly, by improving neovascularization, and indirectly, by enabling more efficient harvesting of EPCs from peripheral blood. The mobilization and incorporation of EPCs can be modulated by recombinant pharmaceuticals and natural hematopoietic or angiogenic stimulants.

Granulocyte macrophage-colony stimulating factor (GM-CSF) induces colony formation in several cell types (e.g., hematopoietic progenitor cells, myeloid lineage cells, bone-marrow stromal cells) and also enhances both the neovascularization of severely ischemic tissues and the *de novo* vascularization of corneas after corneal micropocket surgery. The observed enhancement is believed to evolve from amplified EPC mobilization and increased recruitment of circulating EPCs into the site of new blood vessel formation (Takahashi et al. 1999). In a pilot clinical trial, patients with acute myocardial infarction displayed a significant increase in EPC mobilization after GM-CSF injection (Seiler et al. 2001).

Vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF) (Gehling et al. 2000) are also potent stimulators of EPC mobilization. VEGF is perhaps the most critical growth factor for vasculogenesis and angiogenesis (Carmeliet et al. 1996, Ferrara et al. 1996, Shalaby et al. 1995). The effect of VEGF on EPCs has been observed in mice after intraperitoneal injection of recombinant human $VEGF_{165}$ (Asahara et al. 1999b) and in patients with critical limb ischemia (Kalka et al. 2000a) and myocardial ischemia (Kalka et al. 2000c) who were treated with VEGF gene transfer therapy. Bone marrow-derived EPCs were efficiently mobilized into the circulation by G-CSF injection during placebo-controlled clinical trials in patients who underwent successful primary coronary interventions (Ripa et al. 2006, Zohlnhofer et al. 2006).

Stromal cell-derived factor-1 (SDF-1), which plays an important role in trafficking HSCs, contributes to neovascularization by enhancing the mobilization, recruitment, and proliferation of EPCs (Peichev et al. 2000, Yamaguchi et al. 2003). Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that catalyze a rate-limiting step in cholesterol biosynthesis, enhance EPC mobilization by stimulating the Akt signaling pathway (Dimmeler et al. 2001, Llevadot et al. 2001, Vasa et al. 2001).

EPC Therapy for Ischemic Diseases: Preclinical Studies

Tissue ischemia caused by peripheral, cerebral, and coronary artery disease can be attenuated by the development of collateral circulation, so after the role of EPCs in neovascularization was recognized, investigators began to evaluate the potential therapeutic impact of EPC administration for treatment of ischemic conditions. Kalka et al. (2000b) intravenously administered *ex vivo* expanded EPCs obtained from human peripheral blood into immunodeficient mice with hindlimb ischemia. Recovery of blood flow was greater in mice treated with EPCs than in control mice and mice that received mature endothelial cells, and histological examinations confirmed EPC incorporation and differentiation into endothelial cells. Limb salvage ratio, perhaps the most important endpoint in critical limb ischemia, was also dramatically improved with EPC treatment. Murohara (2001) reported similar benefits with the administration of EPCs isolated from cord blood in a nude rat model of hindlimb ischemia. Experiments performed in our lab evaluated EPC therapy in nude rats after acute myocardial infarction. Intravenous administration of *ex vivo* expanded human EPCs inhibited fibrosis and preserved function, and, as in the hindlimb ischemia model, the infused EPCs were recruited to the ischemic area and contributed to neovascularization (Kawamoto et al. 2001). Freshly isolated (i.e., not cultured) CD34+ EPCs produced similar results in rats with acute myocardial infarction (Kocher et al. 2001), and Taguchi et al. (2004) demonstrated that systemic infusion of human cord blood CD34+ EPCs augmented neovascularization in nude mice after cerebral infarction, thereby enhancing nerve regeneration and contributing to cerebral tissue repair.

The scarcity of EPCs in bone marrow and peripheral blood could limit the utility of EPC infusion in a clinical setting. To increase the effective dose of EPCs, our group administered EPC therapy via local transplantation rather than systemic infusion. In nude rats with acute myocardial ischemia, intramyocardial administration of human circulating CD34+ cells enhanced neocapillary formation, the reduction of infarct size, and functional preservation at a dose equivalent to 5% to 10% of the dose used for systemic infusion (Kawamoto et al. 2003). To simulate the clinical application of EPC transplantation, NOGA electromechanical mapping was used to guide intramyocardial transplantation of autologous EPCs in a swine model of chronic myocardial ischemia; the treatment was associated with significant attenuation of myocardial ischemia as well as functional improvement (Kawamoto et al. 2003).

Bone marrow or peripheral blood MNC preparations that contain a very low concentration (0.2–2%) of EPCs have been reported to enhance ischemic angiogenesis in both preclinical and clinical studies (Tateishi-Yuyama et al. 2002, Tateno et al. 2006). Mononuclear cells other than EPCs (e.g., CD34− cells) can contribute to ischemic neovascularization by secreting angiogenic cytokines (Kamihata et al. 2001); however, the MNCs from bone marrow comprise cells of mesenchymal as well as hematopoietic and endothelial lineages, so administration of the total MNC population could lead to undesirable cellular differentiation and tissue damage. Yoon et al. (2004) reported unexpected and severe myocardial calcification after intramyocardial transplantation of filtered bone marrow cells in a rat model of myocardial infarction. Recently, our lab compared the safety and efficacy of G-CSF-mobilized human CD34+ cells and total MNCs in a nude rat model of acute myocardial infarction. Both treatment groups received the same number of CD34+ cells. Compared to rats treated with purified CD34 + cells, MNC-treated rats displayed a higher incidence of moderate to severe hemorrhagic myocardial infarction accompanied by abundant infiltration of human CD45+ inflammatory cells 3 days after cell transplantation. Four weeks after transplantation, histological capillary density, fibrosis area, and echocardiographic functional parameters were better preserved in the group treated with CD34+ cells than in the MNC-treated group, despite equal dosing of CD34+ cells (Kawamoto et al. 2006a). These results indicate that CD34+ cells possess superior

potency for the preservation myocardial integrity and function after myocardial infarction (Figure 2). Clinical evidence of the superiority of CD34+ cells for myocardial neovascularization has been reported by Hoffman, et al. (2005). The investigators used 3D PET imaging to track infused radiolabeled bone marrow cells that were either unselected or enriched for CD34+. After intracoronary transfer, 14% to 39% of the CD34+ enriched cells were localized to the infarcted myocardium, compared to less than 3% of the unselected bone marrow cells.

The contribution of CD34+ cells to cardiovascular regeneration may not be limited to vascularization. Immunohistochemical and molecular analyses in our laboratory found evidence of CD34+ cell heritage among the cardiomyocytes and smooth muscle cells in the infarcted myocardium of rats (Iwasaki et al. 2006). These findings indicate that the preservation of cardiac function and structural integrity associated with EPC therapy includes both vasculogenic and myogenic components, although the relative contribution of each component has yet to be quantified.

Clinical Application of EPCs for Ischemic Neovascularization

The promising outcomes from preclinical studies of EPC transplantation lead to the initiation of several clinical trials (Table 1). In most trials, EPCs have been isolated from total MNCs via magnetic selection of CD34+ or AC133+ cells. After a pilot study demonstrated the safety and feasibility of the procedure (Stamm et al. 2003), Stamm et al. (2007) found that direct intramyocardial injection of autologous AC133+ cells during coronary artery bypass grafting surgery was associated with a favorable trend for improved cardiac perfusion and left ventricular function. The safety, feasibility, and efficacy of intramyocardial AC133+ cell transplantation has also been established for patients with chronic ischemic cardiomyopathy in the absence of bypass surgery (Klein et al. 2007). Our group performed a phase I/II, randomized, placebo-controlled, dose-ranging, clinical trial to evaluate the intramyocardial transplantation of G-CSF-mobilized CD34+ cells in 24 patients with intractable angina pectoris (Losordo et al. 2007). Patient-specific procedures included G-CSF injection, leukapheresis for cell harvesting, and NOGA mapping-guided cell injection, all of which were well tolerated with no severe adverse events reported. Favorable trends in angina frequency, exercise tolerance, and perfusion defect were observed in patients administered CD34+ cells compared with patients who received placebo. A phase IIb study is underway in the US.

The use of EPC therapy for treatment of acute or subacute myocardial infarction has been investigated by Assmus et al. (2002). Bone marrow total MNCs or an EPC-enriched population of cultured circulating progenitor cells were administered via intracoronary infusion to patients with acute myocardial infarction. Both treatments were associated with improved left ventricular function and regional wall motion. Despite a technical limitation in the culture method regarding EPC purity and the efficiency of expansion, these favorable results encourage future controlled, randomized, clinical trials. Bartunek et al. (2005) demonstrated the efficacy and feasibility of intracoronary AC133+ cell infusion in patients with recent myocardial infarction, although the therapy was associated with an increased incidence of coronary events.

Regarding the EPC application for patients with critical limb ischemia, our group treated 17 patients with intramuscular injection of autologous and G-CSF-mobilized CD34+ cells (Kawamoto et al. 2006b). The cell therapy was safely performed and the final report is now under preparation.

Future Perspectives

A decade has passed since the discovery of adult EPCs in human peripheral blood. During this period, several reports have demonstrated the potency of EPCs in preclinical studies of ischemia and myocardial infarction, and investigators are beginning to evaluate the therapeutic utility of EPCs in early-phase clinical trials. Many trials are likely to recruit patients with critical ischemia who have no other viable treatment options, and it may be difficult to establish significant efficacy in this fragile patient population. As an alternative approach, EPC therapy could be administered after successful primary stenting in patients with acute myocardial infarction; however, these "mild" cases of ischemia or heart failure may not display any notable improvement in prognosis or function with additional therapy. Thus, the success of future clinical investigations of EPC therapy is critically dependent on choosing an appropriate set of inclusion and exclusion criteria. Additional investigations designed to identify the appropriate cell type and dose and to optimize techniques for EPC isolation, expansion, mobilization, recruitment, and transplantation are also needed to continue the advancement of this novel therapeutic modality.

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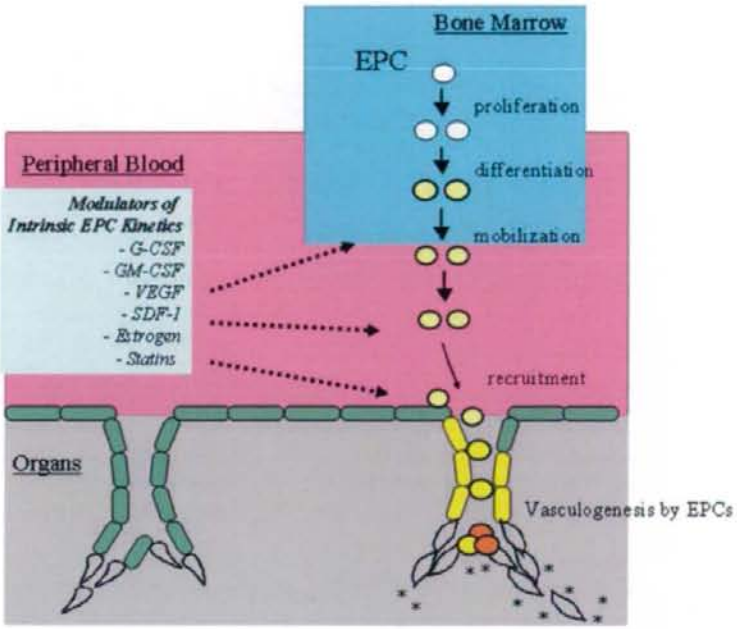

Figure 1.
Mechanism of endothelial progenitor cell (EPC)-mediated postnatal neovascularization. Circulating EPCs mobilized from bone marrow are recruited into the foci of neovascularization and contribute to new blood vessel formation. The intrinsic EPC activity can be augmented by certain cytokines, growth factors, and pharmaceutical agents. G-CSF indicates granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; VEGF, vascular endothelial growth factor; SDF-1, stromal cell-derived factor-1.

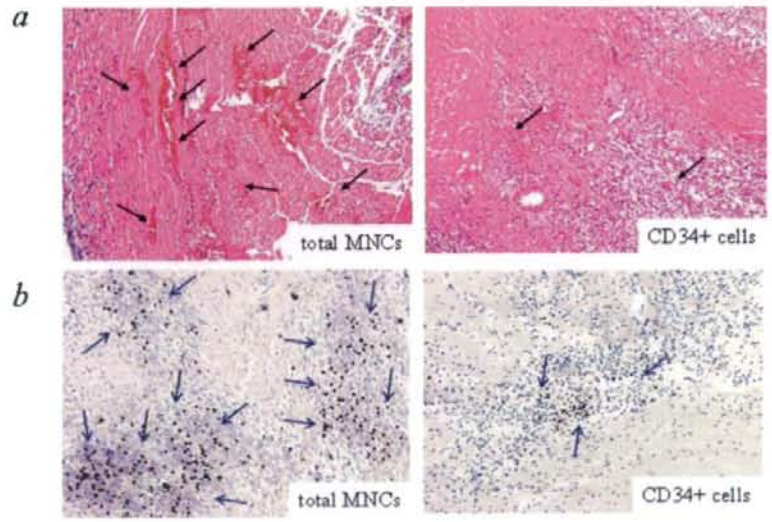

Figure 2.

Representative histological assessments 3 days after intramyocardial injection of CD34+ cells or total mononuclear cells (MNCs). Experiments were performed in nude rats with acute myocardial infarction. Animals in both treatment groups were administered the same number of CD34+ cells. (a) Hematoxylin-eosin staining of the infarct area revealed massive hemorrhagic infarction (arrows) in rats administered total MNCs. (b) Immunostaining for human-specific CD45 to identify hematopoietic/inflammatory cells (arrows) in the infarcted myocardium. Human CD45+ cells were abundant in the total MNC treatment group, but not in the purified CD34+ cell treatment group.

Table 1

Clinical application of endothelial progenitor cells in coronary artery disease.

Study	Patients, n	Cell Type	Disease	Treatment	Control Group	Results
Stamm et al. (2003)	6	BM-AC133+	Chronic MI	IM with CABG	None	LVEF↑ Perfusion↑
Stamm et al. (2007)	40	BM-AC133+	Chronic MI	IM with CABG	CABG	LVEF↑ Perfusion↑
Klein et al.	10	BM-AC133+	Chronic ICM	IM	None	LVEF↑ NYHA class↓
Losordo et al.	24	GCSF-mobilized CD34+ cells	Chronic CAD	IM/NOGA	Placebo	Perfusion↑ CCS class↓
Assmus et al.	20	Cultured CPCs or BM-MNCs	Acute MI	IC	None	LVEF↑ Viability↑
Bartunek et al.	35	BM-AC133+	Recent MI	IC	Standard therapy	LVEF↑ Perfusion↑

BM indicates bone marrow; MI, myocardial infarction; IM, intramuscular injection; CABG, coronary artery bypass graft surgery; LVEF, left ventricular ejection fraction; ICM, ischemic cardiomyopathy; NYHA, New York Heart Association; GCSF, granulocyte colony stimulating factor; CAD, coronary artery disease; CCS, Canadian Cardiovascular Society; CPCs, circulating progenitor cells; IC, intracoronary injection; MNCs, mononuclear cells.

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Review

Circulating endothelial/skeletal progenitor cells for bone regeneration and healing

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Vasculogenesis CD34+ cells

ABSTRACT

An emerging strategy in the regeneration and repair of bone is to use stem cells, including bone marrow mesenchymal stem cells, which are the most investigated and reliable source for tissue engineering, as well as circulating skeletal stem/progenitor cells, which are receiving abundant attention in regenerative medicine due to their ease of isolation and high osteogenic potential. Because failures in fracture healing are largely due to poor vascularization among many environmental factors, we highlight the first proof-of-principle experiments that elucidated the collaborative multi-lineage differentiation of circulating CD34 positive cells – a cell-enriched population of endothelial/hematopoietic progenitor cells – into not only endothelial cells but also osteoblasts. These cells develop a favorable environment for fracture healing via vasculogenesis/angiogenesis and osteogenesis, ultimately leading to functional recovery from fracture. This review will also highlight current concepts of circulating stem/progenitor cell-based therapy and their potential application for bone repair.

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Introduction

Whereas embryonic stem cells in the blastocyst stage can generate into any differentiated cell type, most adult stem cells have a limited potential for postnatal tissue/organ regeneration. Among the phenotypically characterized adult stem/progenitor cells [5,29,54], the hematopoietic system has traditionally been considered as an organized, hierarchical system that is spearheaded by multipotent, self-renewing stem cells at the top, followed by lineage-committed progenitor cells in the middle, and ends with lineage-restricted precursor cells – which give rise to terminally differentiated cells – at the bottom [62]. Recently, however, a new population of stem cells has been added to this schematic, notably, the adult human peripheral blood (PB) CD34+ cells. These cells reportedly contain intensive endothelial progenitor cells (EPCs) as well as hematopoietic stem cells (HSCs) [2], and – as only recently discovered following the discovery of bone marrow (BM)-derived and circulating EPCs in adults – promote embryonic vasculogenesis [1,2,47,50].

A fundamental aspect of tissue engineering research has been to identify various stimuli through which to direct stem cell activity toward tissue regeneration. To this end, recent research has demonstrated that EPCs respond to tissue ischemia as well as cytokines by

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mobilizing from the bone marrow (BM) into PB, ultimately migrating to regions of neovascularization to differentiate into mature endothelial cells and further promote vasculogenesis [57]. As a result of this finding, many researchers have applied EPCs to promote the therapeutic neovascularization in animal models of limb ischemia, myocardial infarction, and liver disorders [3,20,22,24,25,27,28,34,43]. Promising results have particularly been noticed in the immunodeficient rat model of acute myocardial infarction following the transplantation of either human CD34+ cells or EPCs expanded ex-vivo into the site of myocardial neovascularization. Following this implantation, these cells differentiate into mature endothelial cells, augment capillary density, inhibit myocardial fibrosis and apoptosis, and ultimately preserve the left ventricular function [20,24,25,27]. Based on these findings, clinical trial using PB CD34+ cells has been initiated with promising results [30,37,59].

More recent investigations on, promoting tissue neovascularization with EPC has led to broader applications for these cells among various areas of regenerative medicine. Some of these areas include the regeneration of brain tissue, as has been successfully performed by the systemic administration of human cord blood-derived CD34+ cells to immunocompromised mice within 48 h of sustaining a stroke; in these mice, neovascularization was induced along the ischemic zone, providing a favorable environment for neuronal regeneration [56]. Other areas in which PB CD34+ cell transplantation has promoted tissue healing via revascularization include full-thickness skin wounds of diabetic mice [53]. In the latter, our group utilized human cells that were mobilized by the granurocyte colony stinulating factor (G-CSF) contributing to ligament healing via vasculogenesis in the rat medial collateral ligament injury model [58]. Finally, our group has also reported several successful outcomes when utilizing PB CD34+ cells/EPCs for fracture healing [39–41].

In this review, based on our findings and literatures in this fields, we will highlight the concepts of circulating stem/progenitor cell-based therapy and their potential application for bone repair.

Bone and vascularity

Failures in fracture healing are caused due to many systemic and local factors, including immune depression, hormonal milieu, nutrition, mobility, high-energy fracture, extensive soft tissue damage, infection, irradiation, lack of contract between the bone ends, and the actual loss of bone substance. Among them, severe skeletal injuries consisting of fractures with a compromised blood supply result in either delayed unions or established non-unions. An essential requirement for these fractures to heal, then, is to restore the local blood flow, which has traditionally been performed through complex vascular procedures or soft tissue transfers with adequate blood supply in certain types of injuries [16,23]. From the standpoint of regenerative medicine and tissue engineering, neovascularization is accordingly emerging as a bone development and regeneration strategy that appears to benefit quite well from an osteogenic reciprocity that exists between endothelial cells and osteoblasts [23].

The progenitor cell lineages giving rise to endothelial and osteoblastic cells have recently been thought to overlap, contrary to prior beliefs. The data supporting this shifting paradigm has ranged from an analysis of markers for cellular differentiation to data on cell engraftment and isolation from various tissues. *In vitro*, CD34+ and CD133+ cells were not only hematopoietic and vasculogenic, but they were also capable of differentiating into osteoblasts [4,8,60], while *in vivo*, a non-adherent side population of BM cells containing primitive cells was capable of generating into both hematopoietic and osteogenic lineages.[44] Interestingly, purified human hematopoietic/endothelial cells expressing the CD34+ marker have been previously used to generate the antibody Stro-1, which is now widely used to identify mesenchymal stem cells [52]. The combined results from these studies suggest that these hematopoietic/endothelial progenitors may share some phenotypic and perhaps functional (i.e., plutipotency) traits with mesenchymal stem cells, permitting them to give rise to osteoblasts. In a separate series of experiments, a murine BM side population (SP) of cells isolated from murine BM, which contain hematopoietic repopulating cells [17], can engraft in bone after intravenous transplantation [11]; the absence of rejection indicates that these hematopoietic cells may share phenotypic traits that are crucial to prevent immune rejection. Based on these reports, our group sought to confirm the overlapping origin of endothelial and osteogenic markers by running single cell reverse-transcriptase polymerase chain reaction (RT-PCR), and showed that 20% of human peripheral blood CD34+ cells expressed the mRNA for osteocalcin [39].

The presence of the CD34 cell marker among cells with potential for osteogenic differentiation has been further elucidated by other groups. Eghbali-Fatourechi et al. have shown that this marker exists among human circulating osteocalcin or alkaline phosphatase positive cells, and that approximately 40% of osteocalcin positive and 50% of alkaline phosphatase positive cells can be obtained from random blood donors co-expressed CD34., while, conversely, 30% of circulating CD34+ cells co-stain with the osteocalcin antibody [14]. Although several reports suggest that there HSC- or EPC-rich cell populations are committed toward osteogenic differentiation, the morphological and physiological incorporation of these cells for medical application has never been proved until our series [39–41].

As it appears from the above-reviewed studies CD34+ cells are committed to not only endothelial cells but also mural perivascular cells (i.e., pericytes and smooth muscle cells) [20,64]. Similarly, it has been quite recently reported that vascular pericytes may arise from CD34+ cells [19]. In addition to these reports, quite recently, Zengin et al. reported the existence of EPC and stem cells in a distinct zone between smooth muscle and adventitial layer of human adult vascular wall that are capable to differentiate into mature endothelial cells, hematopoietic and local immune cells, such as macrophages [65]. Furthermore, there exists increasing evidence that vascular pericytes are also capable of forming osteoblasts [10], the latter being capable of differentiation into osteoblasts. These findings suggest CD34+ cells may be involved and pooled as vascular progenitor cells in the vascular wall, which transform toward osteogenic lineage in response to various environmental cues.

Circulating BM-derived endothelial/osteoprogenitor cells

Human PB CD34+ cells/EPCs are being investigated to specifically address the problem of delayed and atrophic non-unions in fracture healing, which has a significantly high (5–10%) annual incidence among all long bone fractures and result from an inadequate local blood supply around the zone of injury [38,51]. Because securing an adequate blood supply to this area is crucial for bone healing to occur [9,16], as would be evidenced radiographically by the formation of bridging callus along a former fracture gap, an emerging focus in regenerative medicine is to develop EPCs to promote neoangiogenesis. EPCs are appealing for this task in large part because the link between angiogenesis and the development of native bone on a larger scale has led to the discovery on a cellular level that there exists a developmental reciprocity between endothelial cells and osteoblasts [23]. EPCs are also appealing for this task because a more traditional approach for enhancing the local vascularity along a non-union or delayed union has been to perform vascular bone grafting, which requires painstaking microvascular surgical skills [51].

We first reported that human PB CD34+ cells that were recruited to the fracture site following systemic delivery, developed a favorable environment for fracture healing by enhancing vasculogenesis/angiogenesis and osteogenesis, and finally led to functional recovery from fracture [39] (Fig. 1). Briefly, we systemically transplanted PB CD34+ cells, mononuclear cells (MNCs) or PBS into non-healing femoral fracture model of immunodeficient rats. Bone healing assessed by

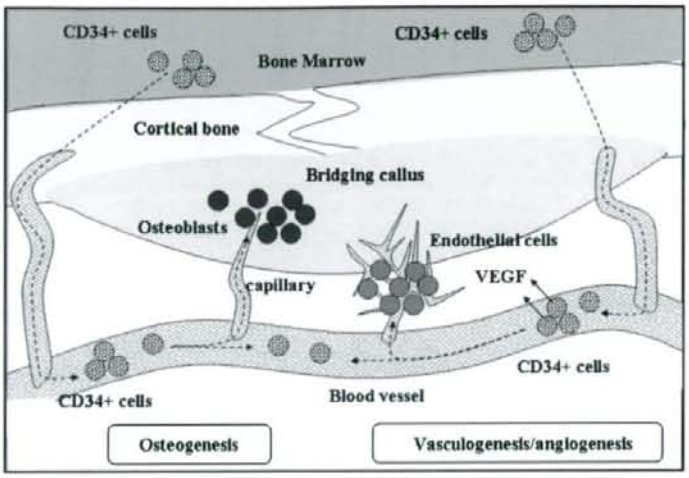

Fig. 1. Human PB CD34+ cells/EPCs, which were systemically transplanted, were recruited to the fracture site, developed a favorable environment for fracture healing by enhancing vasculogenesis/angiogenesis and osteogenesis, and finally led to functional recovery from fracture. In the process of fracture healing, EPCs are mobilized from BM to PB by the trigger of fracture onset.

radiological, histological, biomechanical examinations was significantly enhanced in the CD34+ cell group compared to MNC and PBS group. Laser Doppler imaging demonstrated that fracture-induced ischemia was significantly recovered by CD34+ cell transplantation compared to the other groups. These healing potentials of CD34+ cell transplantation were confirmed as mainly two mechanisms. One is the osteogenic and endothelial differentiation potential of CD34+ cells, and the other is the paracrine effect of CD34+ cells by secreting vascular endothelial growth factor (VEGF). The former was at first confirmed by the recruitment of human cells at the fracture site by labeling cells transplanted, and secondary confirmed by RT-PCR and immunohistochemical staining at the peri-fracture site, which showed molecular and histological expression of human-specific markers for endothelial cells and osteoblasts 2 weeks after CD34+ cell transplantation. The latter one was confirmed by enhanced intrinsic angiogenesis and osteogenesis using rat capillary and osteoblast density at the fracture site in the CD34+ cell group. Interestingly, loss of function test using sFlt1 (VEGF antagonist), following detection of human-specific VEGF expression in the CD34+ cell treated group at the fracture site, showed reduced angio/osteogenesis and fracture healing in the sFlt1 treated group. This first series of study indicated that PB CD 34+ cell transplantation contributed to fracture healing through direct as well as indirect cellular communication in autocrine/paracrine manner; we confirmed the osteogenic properties of these cells via *in vitro* experiments consisting of alizarin red staining and RT-PCR [41]. These findings combined clearly highlight the therapeutic potential of PB CD34+ cells for functional bone healing.

The therapeutic application of circulating CD 34+ cells depends in large part on the availability of these cells in sufficient quantities. Our group has shown that while these cells can efficaciously heal skeletal defects after systemic transplantation, they also migrate to other tissues, including the lung liver, thymus, and brain, raising concerns of unforeseen side effects along these organs; this is particularly true given that large systemic doses may be required for a clinical effect.

In seeking alternative approaches to systemically infusing PB CD34+ cells for promoting skeletal healing, we seeded the femoral non-union site of immunodeficient rats with CD34+ cells that were mobilized with granulocyte colony stimulating factor (G-CSF), and successfully promoted fracture union per radiographic and histologic assessment [41]; our impetus for using G-CSF mobilized cells is that applications in the hematologic and cardiovascular fields have successfully involved the mobilization of EPCs with this factor in order to obtain sufficient therapeutic quantities of PB CD34+ cells [12,29,37]. As expected, bone healing was significantly enhanced by high (10^5) and middle (10^4) dose CD34+ cell transplantation compared to animals receiving low (10^3) dose CD34+ cells and PBS. We confirmed a dose-dependent response on molecular and histological levels via RT-PCR and immunohistochemical staining from tissue around the fracture site, which was most notable via detection of markers for endothelial cells and osteoblasts by 2-week post-transplantatin of CD-34+ cells.

The mechanism by which skeletal healing occurred in these experiments is likely related to dose-dependent vasculo/angiogenic effects of G-CSF-mobilized PB CD34+ cells. In one report, fracture-induced hypoxia and VEGF both upregulate the expression of bone morphogenetic protein (BMP)-2 in microvascular endothelial cells [6]. This is consistent with experimental results from our group in which we show an upregulation of rat VEGF and BMP-2 expression in the microvascular endothelial cells along a rat fracture site. We further showed that PB CD34+ cells have a paracrine effect through which the capillary and osteoblast density increase in a dose-dependent fashion, and we further confirmed the upregulation of rat VEGF and BMP-2 expression around the fracture site. Of note, effective fracture healing occurred as long as there were more than 1×10^4 CD34+ cells implanted along the fracture site. We feel that these findings provide feasible alternatives to current clinical strategies for addressing delayed unions and established non-unions.

While the physiological role of EPCs in fracture healing remains to be clarified, we have begun to characterize the role of mobilized BM-derived EPCs on fracture healing [40] (Fig. 1). We have found that during the early day phase of endochondral ossification, neovascularization peaks by 7 days after a fracture; this was noted via serial Laser Doppler perfusion imaging and histologic quantification of capillary density. Along this site, there is a significant increase in cell populations derived from the infiltrating EPC cells, most notably of BM cKit+ Sca1+Lineage- (Lin-) and PB Sca1+ Lin-cells. The Sca1+ EPC cells contribute to vasculogenesis, as confirmed by double immunohistochemistry for CD31 and Sca1. We further showed that EPCs enhance neovascularization by transplanting BM that transcriptionally express Laz Z from transgenic donors into a fracture into wild type modes; these cells were regulated by the endothelial cell-specific Tie-2 promoter. We then confirmed that EPCs mobilize into a fracture site