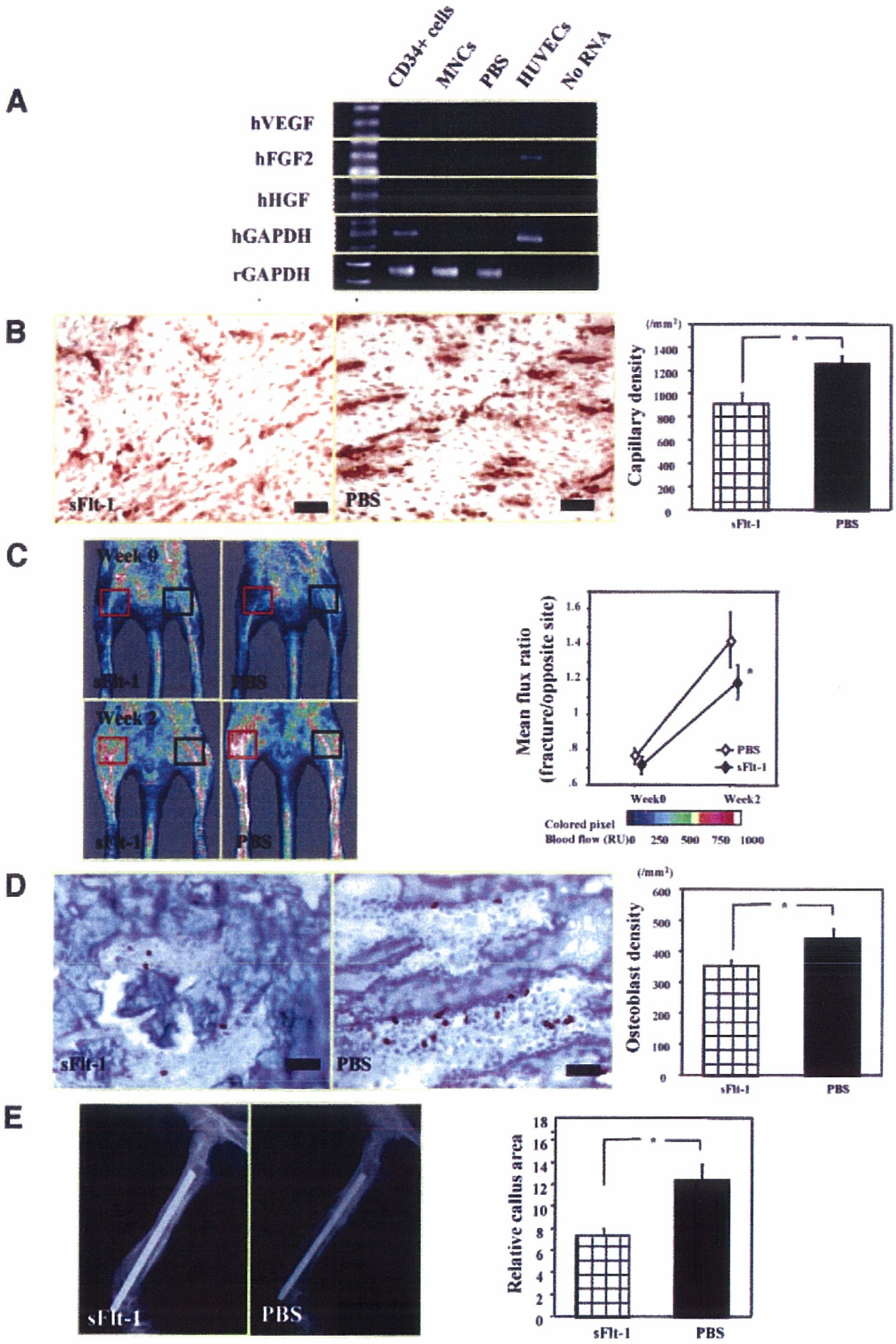
A
CD34+ cells
MNCs
PBS
HUVECs
No RNA
hVEGF
hFGF2
hHGF
hGAPDH
rGAPDH
B
sFlt-1
PBS
Capillary density
(/mm²)
1400
1200
1000
800
600
400
200
0
*
sFlt-1
PBS
C
Week 0
Week 2
sFlt-1
PBS
Mean flux ratio
(fracture/opposite site)
1.6
1.4
1.2
1
.8
.6
*
PBS
sFlt-1
Week0
Week2
Colored pixel
Blood flow (RU)0 250 500 750 1000
D
sFlt-1
PBS
Osteoblast density
(/mm²)
600
500
400
300
200
100
0
*
sFlt-1
PBS
E
sFlt-1
PBS
Relative callus area
18
16
14
12
10
8
6
4
2
0
*
sFlt-1
PBS

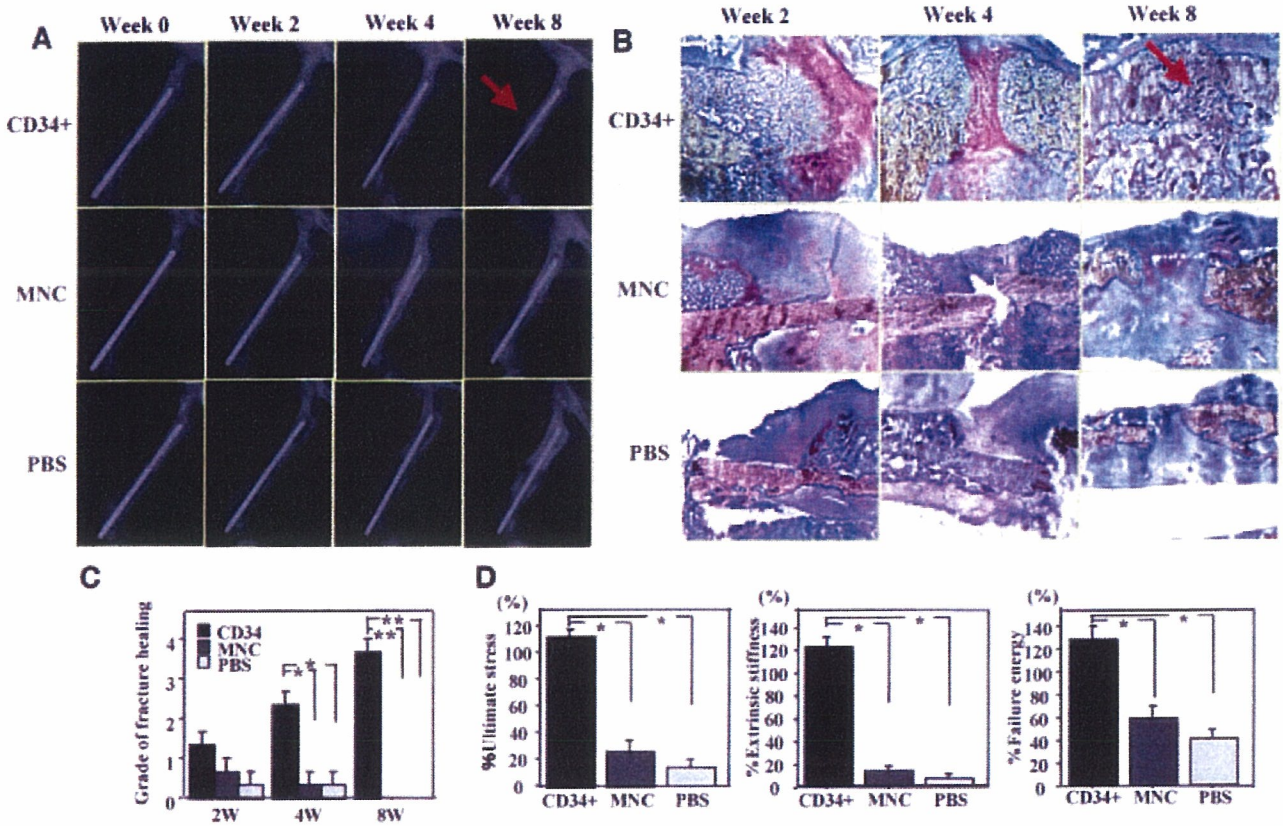

Figure 7. Radiographical, histological, and biomechanical evidence of fracture healing after CD34$^+$ cell transplantation. **A:** Fracture healing was serially assessed by radiographs. By week 8, fracture was healed with bridging callus in all animals receiving CD34$^+$ cells (**arrow**), but in no rats treated with MNCs or PBS alone. **B:** Serial assessment of fracture healing by histological examination with toluidine blue staining. Histological evaluation demonstrated the enhanced endochondral ossification consisting of numerous chondrocytes and newly formed trabecular bone at week 2, bridging callus formation at week 4, and complete union at week 8 (**arrow**) in animals receiving CD34$^+$ cell transplantation. Although thick callus formation at week 2 was observed, the healing process had stopped by week 4, and the callus was finally absorbed at week 8 in animals receiving MNCs or PBS. **C:** The degree of fracture healing was assessed by Allen's classification. The degree of fracture healing at weeks 4 and 8 was significantly higher after CD34$^+$ cell transplantation compared with other treatments. $^*P < 0.05$; $^{**}P < 0.01$. **D:** Functional recovery after fracture is assessed by biomechanical three-point bending test at week 8. The percentage of all parameters (percent ultimate stress, percent extrinsic stiffness, percent failure energy) showing the ratio of each value in fractured side with contralateral side in animals receiving CD34$^+$ cells was significantly superior to those in animals receiving MNCs or PBS. $^*P < 0.05$.

compared with the other groups (CD34$^+$, 13.67 ± 1.76; MNC, 7.00 ± 0.57; PBS, 5.00 ± 1.15, respectively; $P <$ 0.05 for CD34$^+$ versus MNC or PBS group) (Figure 5D). These results indicate that administration of human peripheral blood CD34$^+$ cells contribute to improvement of tissue perfusion and enhancement of bone formation after fracture.

Inhibition of Intrinsic Angiogenesis and Osteogenesis by Anti-Angiogenic Agent in Animals Receiving CD34$^+$ Cell Transplantation

At first, we confirmed that transplanted human CD34$^+$ cells released a greater amount of angiogenic factors (hVEGF, hFGF2, hHGF) than the MNCs at the fracture site (Figure 6A). Next, to investigate the hypothesis that neovascularization is essential to support endogenous bone regeneration after CD34$^+$ cell infusion, we used an anti-angiogenic agent, sFlt1. Rat-specific vascular staining with isolectin B4 at week 2 demonstrated reduced capillary density in zone a in animals treated with CD34$^+$ cells and sFlt1 compared with those receiving CD34$^+$ cells and PBS (sFlt1, 908.3 ± 90.6/mm^2; PBS, 1258.3 ± 69.1/mm^2, respectively; $P < 0.05$ for sFlt1 versus PBS group) (Figure 6B). The skin blood flow within the fractured site and the intact contralateral site were calculated by LDPI. By week 2, the ratio of fractured/intact contralateral blood flow was significantly reduced in the sFlt1-

Figure 6. Inhibition of intrinsic angiogenesis and osteogenesis by anti-angiogenic agent in animals receiving CD34$^+$ cell transplantation. **A:** RT-PCR revealed that fractured tissue sample contained higher amount of angiogenic factors (hVEGF, hFGF2, and hHGF) in human CD34$^+$ group compared with MNC group. **B:** Rat-specific vascular staining with isolectin B4 (brown) at week 2 demonstrated reduced neovascularization in zone a in animals treated with CD34$^+$ cells and sFlt1 compared with those receiving CD34$^+$ cells and PBS. Intrinsic angiogenesis assessed by capillary density at week 2 is significantly reduced after sFlt1 treatment compared with PBS. $^*P < 0.05$. **C:** Representative LDPI at weeks 0 and 2 in each group (**red square**, fracture site; **black square**, intact contralateral site). Mean flux ratio at week 2 was significantly less in animals treated with sFlt1 compared with rats receiving PBS. $^*P < 0.05$. **D:** Representative rat-specific OC staining (brown) to detect intrinsic OBs at week 2 in zone b in animals treated with CD34$^+$ cells and sFlt1 and those receiving CD34$^+$ cells and PBS. Intrinsic osteogenesis assessed by the OB density at week 2 was significantly inhibited after sFlt1 treatment compared with PBS. $^*P < 0.05$. **E:** Relative callus area assessed by radiograph at week 2 was significantly smaller in sFlt1-treated group compared with PBS group. $^*P < 0.05$. Original magnifications, ×200.

treated group compared with the PBS group (sFlt1, 1.180 ± 0.096; PBS, 1.428 ± 0.159, respectively; $P < 0.05$ for sFlt1 versus PBS group) (Figure 6C).

Rat-specific OC staining at week 2 revealed a reduction of OB density, a parameter of intrinsic osteogenesis, in zone b in animals treated with $CD34^+$ cells and sFlt1 compared with those receiving $CD34^+$ cells and PBS (sFlt1, 352.0 ± 17.3/mm^2; PBS, 440.7 ± 30.0/mm^2, respectively; $P < 0.05$ for sFlt1 versus PBS group) (Figure 6D). The relative callus area assessed by radiograph at week 2 was significantly smaller in sFlt1-treated group compared with PBS group (sFlt1, 7.33 ± 0.67; PBS, 12.33 ± 1.53, respectively; $P < 0.05$ for sFlt1 versus PBS group) (Figure 6E). These findings indicate that the administration of human $CD34^+$ cells contributed in an autocrine/paracrine manner to fracture healing by releasing angiogenic factors.

Morphological and Functional Recovery of Fractured Bone in Animals Receiving $CD34^+$ Cell Transplantation

Morphological recovery of fractured bone in each group was evaluated by radiographical and histological examinations. In 66% of the rats (9 of 12) at week 4 and all rats at week 8 (nine of nine) in the $CD34^+$ group, the fracture radiographically healed with bridging callus formation, whereas the fracture site in the other groups showed no bridging callus formation and resulted in nonunions, consistent with a previous report showing the natural course of this animal model (Figure 7A).[20] Histological evaluation with toluidine blue staining demonstrated enhanced endochondral ossification consisting of numerous numbers of chondrocytes and newly formed trabecular bone at week 2, bridging callus formation at week 4, and complete union at week 8 in the $CD34^+$ group (Figure 7B). In contrast, although a thick callus formation was observed at week 2, the healing process had stopped by week 4 and finally the callus was absorbed at week 8 in the other groups (Figure 6B). The degree of fracture healing assessed by Allen's classification[25] was significantly higher in $CD34^+$ group compared with other groups at weeks 4 and 8 (2 weeks: $CD34^+$, 1.3 ± 0.3; MNC, 0.7 ± 0.3; PBS, 0.3 ± 0.3, not significant for $CD34^+$ versus other groups; 4 weeks: $CD34^+$, 2.3 ± 0.3; MNC, 0.3 ± 0.3; PBS, 0.3 ± 0.3, $P < 0.05$ for $CD34^+$ versus other groups; 8 weeks: $CD34^+$, 3.7 ± 0.3; MNC, 0.0 ± 0.0; PBS, 0.0 ± 0.0, $P < 0.01$ for $CD34^+$ versus other groups, respectively.) (Figure 7C).

Furthermore, to confirm the functional recovery of the fractured bone, biomechanical evaluation by a three-point bending test was performed at week 8 in all groups. Specimen length was similar in $CD34^+$ cell group (15.2 ± 1.9 mm), MNC group (15.5 ± 2.1 mm), and PBS group (14.8 ± 1.3 mm). The percent ratios of all parameters in the fractured femur versus contralateral intact femur in $CD34^+$ group were significantly superior to those in the other groups (percent ultimate stress: $CD34^+$, 112.3 ± 5.3; MNC, 25.5 ± 8.3; PBS, 13.1 ± 4.3%, $P < 0.01$ for $CD34^+$ versus other groups; percent extrinsic stiffness: $CD34^+$, 121.1 ± 8.1; MNC, 12.1 ± 3.8; PBS, 6.8 ± 2.6%, $P < 0.01$ for $CD34^+$ versus other groups; percent failure energy: $CD34^+$, 127.3 ± 12.1; MNC, 57.3 ± 10.7; PBS, 40.3 ± 8.2%, $P < 0.05$ for $CD34^+$ versus other groups) (Figure 7D). These results indicate that the femoral fractures with cauterized periosteum in the immunodeficient rats were morphologically and functionally healed by the administration of human peripheral blood $CD34^+$ cells.

Discussion

Severe skeletal injury accompanied by fracture and loss of blood supply results in delayed union or established nonunion. Recovery of blood flow at the injury site is considered to be essential for adequate fracture healing.[15,16] Therefore, appropriate neovascularization is emerging as a prerequisite for bone development and regeneration through developmental reciprocity between ECs and OBs.[16] One of the most promising approaches for overcoming this clinical issue is by grafting the vascularized bone; however, this process requires painstaking microvascular surgical skill.[12] Thus, the development of a more practical strategy for fracture healing is required, and many surgeons have begun looking at therapeutic neovascularization to answer this need. Recent advances in stem cell biology have suggested the feasibility and effectiveness of cell-based therapy. Human circulating $CD34^+$ cells, EPC- and HSC-enriched fraction, have been shown to have therapeutic potential for ischemic diseases through vasculogenesis mechanism in our previous studies.[5–7] As we anticipated, mouse $Scal^+Lin^-$ cells, quite similar to human $CD34^+$ cells, were mobilized into peripheral blood in the natural course of fracture healing and human $CD34^+$ transplantation induced significant vasculogenesis in regenerating tissues and enhanced functional recovery from nonhealing fracture in small animal models.

However, human $CD34^+$ cells are reported to be not only hematopoietic and vasculogenic but also capable of differentiating into OBs *in vitro*.[17–19] Quite recent reports demonstrated that the murine BM side population (SP) cells, which contain hematopoietic repopulating cells,[36] can engraft in bone after intravenous transplantation[37] and that a nonadhesive population of cultured BM cells contains primitive cells capable of generating into both hematopoietic and osteogenic lineages *in vivo*.[38] In addition, Ford and colleagues[20] recently reported that $CD34^+$ osteoblastic cells line the cavities of the cartilage in the fracture site of rabbit tibial osteotomy model. Although several reports suggest the commitment of HSC- or EPC-rich cell population into osteogenic lineage cells, the morphological and physiological incorporation of these cells for medical application has never been proved.

In the present study, we used a reproducible animal model of nonhealing 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 delayed union or nonunion. We identified *in vivo* multilineage plasticity of human periph-

eral blood CD34+ cells into OBs as well as ECs by not only immunohistochemistry but also by RT-PCR for human-specific cell markers. Recent reports demonstrated *in vitro* or *in vivo trans*-lineage differentiation of CD34+ cells into cardiomyocytes,[39–42] and peripheral blood CD34+ cells were also shown to contain a cell fraction expressing not only hematopoietic and endothelial but also cardiac, skeletal, hepatic, and neural lineage markers after G-CSF administration or myocardial ischemia.[43,44] However, the exact mechanism of the phenomena is not yet fully understood. Considering that CD34+ cells are EPC and HSC enriched but still a heterogeneous cell population, there may be several possible mechanisms underlying the multilineage differentiation: 1) differentiation of original multipotent stem cells into multiple lineages, 2) differentiation of multiple kinds of lineage-committed progenitor cells into each lineage, 3) transdifferentiation of hematopoietic/endothelial lineage cells into mesenchymal lineage, and 4) cell fusion between transplanted cells and recipient cells. The present RT-PCR results revealed a weak gene expression of human-specific OC in freshly isolated human CD34+ cells, strongly supported by single-cell PCR analysis (before transplantation) and enhancement of the gene expression 14 days after transplantation. These findings indicate that CD34+ cell fraction contains a few multipotent stem and/or committing osteogenic progenitor cells, subsequently representing the osteogenic activity under the microenvironment of fracture. Enhbali-Fatourechi and colleagues[45] quite recently demonstrated that sorted OC-positive cells in human peripheral blood formed mineralized nodules *in vitro* and bone in an *in vivo* transplantation assay. Their novel report may support the hypothesis that CD34+ cell fraction contains a few osteo/endothelial progenitor cells, contributing to an enhanced functional recovery of the fracture.

In addition to these sensitive assessments of donor cell differentiation at the incorporated site, quantitative histochemical analysis for rat ECs and OBs revealed the enhancement of intrinsic angiogenesis and osteogenesis by recipient cells after the administration of human CD34+ cells. Human CD34+ cells were reported to secrete numerous angiogenic factors *in vitro*, including VEGF, HGF, FGF2, and IGF1.[46,47] Our *in vivo* data also demonstrated that gene expression levels of human angiogenic factors including VEGF, FGF2, and HGF at the fracture site were greater in the CD34+ cell group compared with the MNC group. Moreover, we demonstrated that the inhibition of angiogenesis by sFlt1 suppressed not only angiogenesis/vasculogenesis but also intrinsic osteogenesis, indicating that angiogenic factors released by the transplanted CD34+ cells contribute, at least in part, to fracture healing in a paracrine manner. Although the detailed mechanism of the paracrine effect of human CD34+ cells is still unclear, a cooperative signal between HSCs/EPCs and OBs may need to be further considered as a mechanism of multilineage regeneration by CD34+ cell transplantation. The niche regulating birth and differentiation of HSCs is known to consist of OBs, which line the inner surface of BM.[48,49] Zhang and colleagues[50] reported that depleting the receptor of bone morphogenic protein in OBs caused a doubling in both OB and HSC populations, offering some insight into the native of HSCs. Calvi and colleagues[51] found the parallel expansion of HSCs when the number of OBs increased by parathyroid hormone infusion. In addition, Ponomaryov and colleagues[52] reported that immature OBs and ECs control homing, retention, and repopulation of SRC (severe combined immunodeficiency repopulating cells) by secreting SDF-1 as a host defense in response to DNA damage. These findings indicate that osteogenesis and hematopoiesis/vasculogenesis closely regulate each other in terms of microenvironmental interaction for regenerative activity in BM. Microenvironmental interaction between osteogenic and vasculogenic lineage cells may involve not only paracrine regulatory factors but also direct cellular communications in developing CD34+ cells. Enhanced angiogenesis/vasculogenesis signals could exert a cellular commitment and promote development of CD34+ cells into the osteogenic lineage in response to a rigorous demand for skeletal tissue repair as a cooperative organogenesis mechanism.

The present results, showing the potential of CD34+ cells for multilineage plasticity, suggest that CD34+ cell transplantation contributes to forming an ideal local environment for fracture healing by supplying adequate blood flow and stimulating osteogenesis. The environmental contribution of CD34+ cells resulted in morphological and physiological healing of the fracture. Radiographical examination and histological toluidine blue staining demonstrated enhancement of endochondral ossification leading to bridging callus formation only after CD34+ cell transplantation, but this did not occur in the MNC and PBS groups. Finally, the biomechanical three-point bending test confirmed significant functional recovery from fracture after administration of CD34+ cells. These findings strongly suggest that peripheral blood CD34+ cells have significant potential for therapeutic application to the damaged skeletal tissue.

On the basis of preclinical achievements of cell-based therapy for injured bone[53] or osteogenesis imperfecta,[54] transplantation of whole BM cells or BM mesenchymal stem cells has been clinically applied for bone/cartilage regeneration.[55,56] It remains to be clarified whether circulating CD34+ cell transplantation is superior to the BM cell therapy in terms of efficacy and safety for fracture healing. However, cell harvest from peripheral blood dose provides positives in that it is less invasive and safer than BM aspiration under general anesthesia, and magnetic sorting of CD34+ cells has been clinically applied in the hematology field for many years.[57,58] The technical feasibility in a clinical situation and the present preclinical findings demonstrating morphological and functional fracture healing through concurrent vasculogenesis and osteogenesis strongly suggest promising results for the future clinical application of circulating CD34+ cells for nonhealing fracture.

In conclusion, human circulating CD34+ cells have potent vasculogenic and osteogenic plasticity in the fracture-induced environment, enabling them to make a remarkable contribution to morphological and functional bone healing.

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Estradiol Enhances Recovery After Myocardial Infarction by Augmenting Incorporation of Bone Marrow–Derived Endothelial Progenitor Cells Into Sites of Ischemia-Induced Neovascularization via Endothelial Nitric Oxide Synthase–Mediated Activation of Matrix Metalloproteinase-9

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Background—Recent data have indicated that estradiol can modulate the kinetics of endothelial progenitor cells (EPCs) via endothelial nitric oxide synthase (eNOS)–dependent mechanisms. We hypothesized that estradiol could augment the incorporation of bone marrow (BM)–derived EPCs into sites of ischemia-induced neovascularization, resulting in protection from ischemic injury.

Methods and Results—Myocardial infarction (MI) was induced by ligation of the left coronary artery in ovariectomized mice receiving either 17β-estradiol or placebo. Estradiol induced significant increases in circulating EPCs 2 and 3 weeks after MI in estradiol-treated animals, and capillary density was significantly greater in estradiol-treated animals. Greater numbers of BM-derived EPCs were observed at ischemic sites in estradiol-treated animals than in placebo-treated animals 1 and 4 weeks after MI. In eNOS-null mice, the effect of estradiol on mobilization of EPCs was lost, as was the functional improvement in recovery from acute myocardial ischemia. A decrease was found in matrix metalloproteinase-9 (MMP-9) expression in eNOS-null mice under basal and estradiol-stimulated conditions after MI, the mobilization of EPCs by estradiol was lost in MMP-9–null mice, and the functional benefit conferred by estradiol treatment after MI in wild-type mice was significantly attenuated.

Conclusions—Estradiol preserves the integrity of ischemic tissue by augmenting the mobilization and incorporation of BM-derived EPCs into sites of neovascularization by eNOS-mediated augmentation of MMP-9 expression in the BM. Moreover, these data have broader implications with regard to our understanding of the role of EPCs in post-MI recovery and on the sex discrepancy in cardiac events. (***Circulation.* 2006;113:1605-1614.**)

Key Words: angiogenesis ■ endothelium ■ myocardial infarction ■ nitric oxide synthase ■ stem cells

Adult organs exhibit virtually no angiogenesis under normal conditions, except in the female reproductive tract. Several lines of experimental evidence have demonstrated that ovarian sex steroid hormones, such as estrogen and progesterone, modulate angiogenesis via effects on endothelial cells. Estradiol induces endothelial proliferation and migration[1] mediated by the classic estrogen receptor, which is expressed by endothelial cells.[2–4] Previously, endometrial neovascularization throughout the menstrual cycle has been considered to be the result of angiogenesis, ie, proliferation and migration of fully differentiated endothelial cells from preexisting "parent" vessels.[5,6] However, normal monthly physiological endometrial proliferation would require that endothelial cells in the uterus replicate >1000 times during the reproductive life span of the average human female. Accordingly, it is unlikely that differentiated endothelial cells in situ could accomplish this mission without the occurrence of replicative senescence.[7]

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Recently, endothelial progenitor cells (EPCs) isolated from peripheral blood have been shown to incorporate into foci of

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Hemodynamic Parameters in Mice Receiving Estrogen or Placebo

			After MI			
	n	Before MI	1 wk	2 wk	3 wk	4 wk
LVDd, mm						
Estrogen	15	2.8±0.03	3.1±0.04	3.9±0.07	3.7±0.06*	3.8±0.04*
Placebo	10	2.8±0.03	3.0±0.04	4.0±0.12	4.0±0.05	4.2±0.06
LVDs, mm						
Estrogen	15	1.4±0.03	1.9±0.04	2.8±0.09	2.5±0.04*	2.6±0.05*
Placebo	10	1.4±0.02	1.8±0.03	3.0±0.11	3.0±0.08	3.2±0.06
FS, %						
Estrogen	15	52±0.4	38±1.0	30±1.7	31±0.5*	32±0.7*
Placebo	10	52±0.6	39±0.7	25±1.2	24±0.9	23±1.2
HR, bpm						
Estrogen	15	478±13	511±12	532±6	502±10	492±8*
Placebo	10	487±13	517±16	565±11	542±12	562±10
LVSP, mm Hg						
Estrogen	6	...	62±3.1	...	...	74±2.7
Placebo	6	...	62±1.5	...	...	67±2.4
LVEDP, mm Hg						
Estrogen	6	...	1.4±0.2	...	...	1.6±0.4
Placebo	6	...	2.8±0.6	...	...	5.4±1.9
LV +dP/dt, mm Hg/s						
Estrogen	6	...	3094±117	...	...	2984±155†
Placebo	6	...	2856±79	...	...	2252±180
LV −dP/dt, mm Hg/s						
Estrogen	6	...	2550±85	...	...	2122±107*
Placebo	6	...	2081±183	...	...	1650±83

Values are mean±SEM. FS indicates fractional shortening; HR, heart rate; LVSP, left ventricular systolic pressure; and LVEDP, left ventricular end-diastolic pressure.

*$P<0.01$ and †$P<0.05$ vs placebo group.

neovascularization in the adult; this is consistent with the notion of postnatal vasculogenesis.[8,9] These circulating EPCs are derived from bone marrow (BM) and are mobilized endogenously in response to tissue ischemia or exogenously by cytokine stimulation.[10] Previous findings from our laboratory[11] have suggested that cyclic neovascularization of the endometrium involves estradiol-regulated in situ incorporation and differentiation of BM-derived EPCs and that estradiol could also augment the recruitment of EPCs for vascular repair.[12,13] These prior studies have also suggested that estradiol exerts its effects on EPCs via an endothelial nitric oxide synthase (eNOS)–dependent mechanism. The effect of estradiol on vascular repair of the heart, and in particular the potential role of EPCs in this process, has not been previously investigated. Accordingly, in the present study, we investigated the hypothesis that estradiol may augment EPC incorporation into sites of myocardial neovascularization after myocardial infarction (MI).

Methods

A detailed Methods section is provided in the online Data Supplement available at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.553925/DC1.

Statistical Analysis

All values are expressed as mean±SEM. Statistical significance was evaluated using unpaired Student *t* tests for comparisons between estradiol- and placebo-treated mice. When multiple time-point measurements were taken over time, repeated-measures analysis was performed, followed by an unpaired *t* test. Differences in mortality rates were compared by using Fisher exact tests. A value of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the article as written.

Results

Estradiol Preserves Left Ventricular Function After Acute MI

MI was induced in ovariectomized mice receiving either estradiol or placebo via a subcutaneously implanted pellet.[13] Left ventricular (LV) function and dimensions (LV diastolic dimension [LVDd], LV systolic dimension [LVDs], fractional shortening, and heart rate) were similar in estradiol- and placebo-treated mice before and for the first 2 weeks after MI (Table). Beginning 3 weeks after MI, however, echocardiography revealed less ventricular dilation in the estradiol-treated versus placebo-treated mice (LVDd, 3.7±0.06 versus 4.0±0.05 mm, respectively, at 3 weeks, and 3.8±0.04 versus

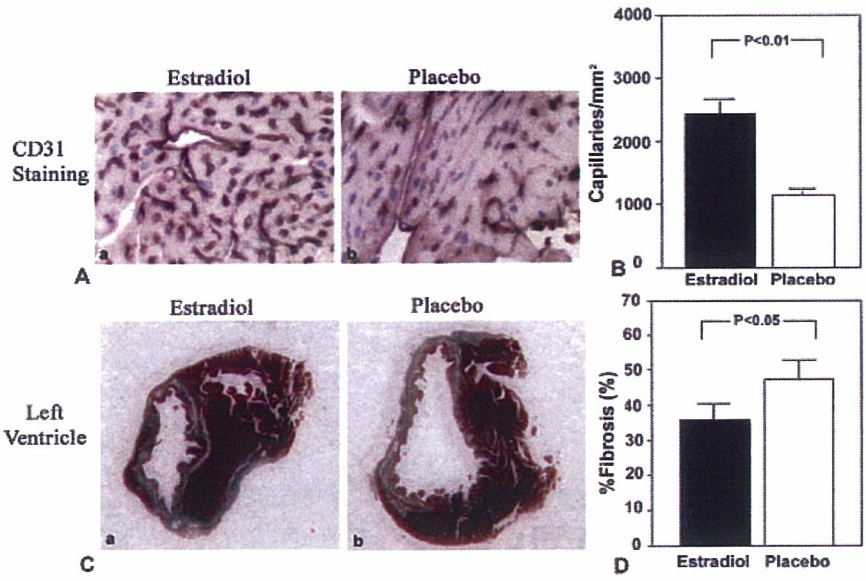

Figure 1. Estradiol increases capillary density and reduces fibrosis after MI. A, Shown are immunohistochemical findings in the peri-infarct myocardium after use of an antibody against isolectin B4 at 4 weeks after MI (a indicates estradiol group; b, placebo group; original magnification ×200). B, Capillary density is shown in peri-infarct myocardium 4 weeks after MI in mice receiving estradiol (n=15) or placebo (n=10). C, Elastic tissue/trichrome-stained myocardium 4 weeks after MI from mice receiving estradiol (a) or placebo (b) shows decreased fibrosis with estradiol treatment. D, Ratio of fibrosis area to LV area in estradiol (n=15) and placebo (n=10) groups reveals decreased fibrosis (scarring) after MI with estradiol treatment.

4.2±0.06 mm, respectively, at 4 weeks [$P<0.01$]; LVDs, 2.5±0.04 versus 3.0±0.08 mm, respectively, at 3 weeks and 2.6±0.05 versus 3.2±0.06 mm, respectively, at 4 weeks [$P<0.01$]). LV function was also significantly better in the estradiol group than in the placebo group (fractional shortening, 31±0.5 versus 24±0.9 mm, respectively, at 3 weeks and 32±0.7 versus 23±1.2 mm, respectively, at 4 weeks [$P<0.01$]). Finally, heart rate, an excellent prognostic indicator after MI, was lower (better) in the estradiol group than in the placebo group (492±8 versus 562±10 bpm, respectively [$P<0.01$]).

Hemodynamic measurements were performed 1 and 4 weeks after MI (Table). There was no significant difference in LV systolic or end-diastolic pressure between the groups at either time point after MI. However, both LV +dP/dt and LV −dP/dt, sensitive indicators of LV function, were significantly better preserved in the estradiol vs the placebo group 4 weeks after MI (LV +dP/dt, 2984±155 versus 2252±180 mm Hg/s, respectively [$P=0.01$]; LV −dP/dt, 2122±107 versus 1650±83 mm Hg/s, respectively [$P<0.01$]).

Serum levels of 17β-estradiol in the placebo group 1 and 4 weeks after MI were <2 pg/mL, whereas levels in the estradiol group after 1 and 4 weeks after MI were 580±55 and 559±62 pg/mL, respectively; these values are consistent with the upper range of levels in premenopausal females.[13]

Acute mortality within 1 week after MI in the estradiol groups (mortality rate, 19%; total number of operated mice=48, including 36 wild-type [WT] and 12 Tie-2/LacZ [LZ]/bone marrow transplant [BMT] mice) was similar to that in the placebo group (mortality rate, 15%; total number of operated mice=40, including 31 WT and 9 Tie-2/LZ/BMT mice).

Histological Assessment of WT Animals

Capillary density 4 weeks after MI was significantly greater in the estradiol group than in the placebo group (2432±110/mm² versus 1134±39/mm², respectively [$P<0.01$]; Figure 1A and 1B). The finding of preserved/improved capillary density has been associated with improved physiological outcome in multiple animal models and has been correlated with a decreased incidence of heart failure.[14]

Elastic trichrome–stained tissue in the placebo group also indicated marked dilatation of the LV cavity, which is consistent with the echocardiographic measurements. In comparison, the estradiol group showed significantly less LV remodeling (Figure 1C). Moreover, the area of LV fibrosis, indicating the zone of permanent myocardial injury, was significantly less in mice receiving estradiol than in the placebo group (36.0±2.6% versus 47.5±2.4%, respectively [$P<0.05$]; Figure 1D). These findings of preserved chamber dimensions (less dilation) and decreased LV fibrosis represent positive long-term prognostic findings.[15,16]

Effect of Estradiol on Circulating EPC Kinetics After Acute MI

As shown in Figure 1A, a greater number of BM-derived EPCs, identified by double staining for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)–acetylated LDL and Bandeiraea simplicifolia-1 lectin, were observed 2 and 3 weeks after MI in the estradiol group than in the placebo group. It is notable that the stimulus of MI alone resulted in a significant increase in circulating EPCs in both groups in the first week after MI, indicating that mobilization of these cells is a natural response to myocardial injury. Indeed, this has been documented in humans as well,[17] with enhanced mobilization of EPCs correlated with better long-term outcome.[18] Within 1 week after MI, however, the number of EPCs decreased in a time-dependent manner in the placebo group (Figure 2B), whereas the estradiol group maintained a significantly higher number of circulating EPCs for 2 and 3 weeks after MI (2 weeks, 430±56/mm² versus 212±19/mm² for estradiol versus placebo groups, respectively; 3 weeks, 265±25/mm² versus 161±17/mm² for estradiol versus placebo groups, respectively [$P<0.01$]). These findings were corroborated by fluorescence-activated cell sorter (FACS) analysis of peripheral blood samples collected 1, 2, and 4 weeks after MI, indicating that the number of

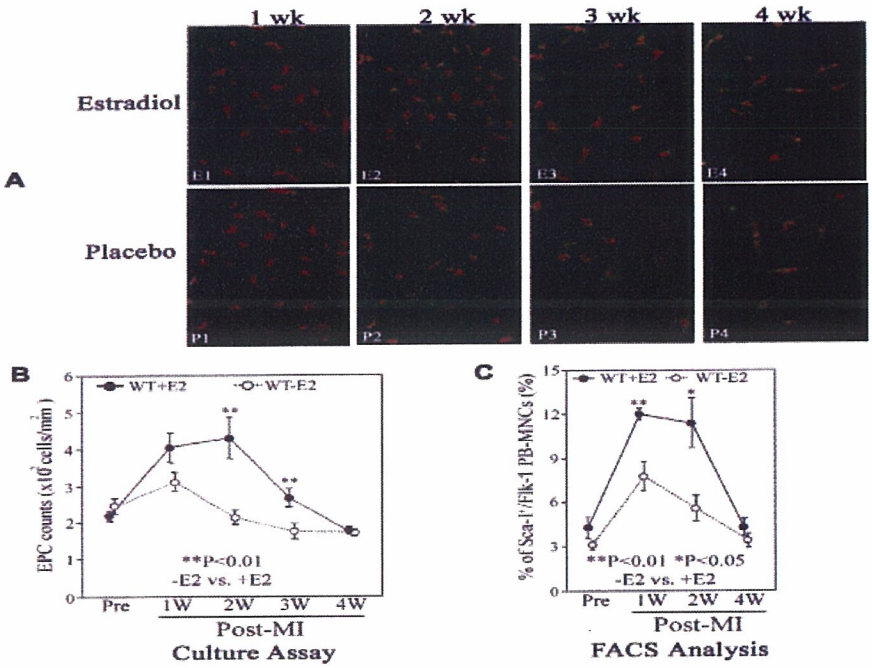

Figure 2. Estradiol augments EPC kinetics after MI. A, Representative immunofluorescent staining of EPCs cultured from mouse peripheral blood at serial time points after MI. Circulating mononuclear cells (PB-MNCs) were collected, and immunofluorescent staining for isolectin B4 was performed, followed by labeling with DiI-acetylated LDL, both of which identify endothelial cells. Double-positive cells are counted as EPCs (E1 through E4 indicates estradiol group at 1, 2, 3, and 4 weeks, respectively, after MI; P1 through P4, placebo group at 1, 2, 3, and 4 weeks, respectively, after MI; magnification ×200). B and C, Circulating EPC counts at serial time points before and after MI in estradiol (+E2) and placebo (−E2) groups assessed by culture assay (B) and FACS for Sca-1 and Flk-1 expression (C) (n=6 in each group at each time point).

Sca-1$^+$/Flk-1$^+$ cells was consistently greater in the estradiol-treated mice than in placebo-treated animals (Figure 2C).

Enhanced Contribution of BM-Derived EPCs to Myocardial Neovascularization

Next, Tie-2/LZ/BMT mice were euthanized 1 and 4 weeks after MI. As shown in Figure 3, macroscopic examination of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)–stained hearts 1 week after MI revealed evidence of abundant recruitment of BM-derived progenitor cells, indicated by the blue areas in the cross sections of the myocardium. Four weeks after MI, macroscopic examination the LVs suggested that a significantly greater number of X-gal–positive cells were present in the estradiol-treated mice than in the placebo-treated mice (Figure 3). Microscopic examination revealed

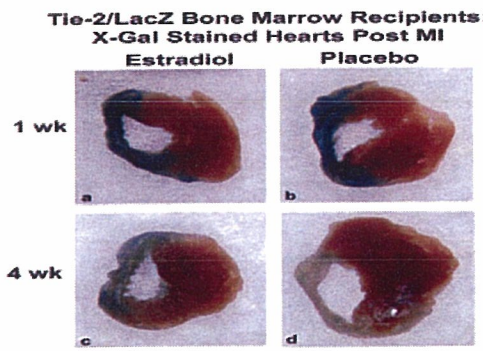

Figure 3. Estradiol increases recruitment of BM-derived EPCs to the myocardium after MI. Representative photographs of X-Gal–stained hearts from WT mice that were recipients of BM from Tie-2/LacZ donor mice. One week after MI, there is abundant blue staining in hearts from estradiol- and placebo-treated mice, indicating acute recruitment of BM-derived Tie-2–expressing cells. After 4 weeks, the blue color is visibly greater in the myocardium of the estradiol-treated mouse (a and c indicate estradiol at 1 and 4 weeks after MI; b and d, placebo at 1 and 4 weeks after MI).

that at 1 and 4 weeks after MI, the number of X-gal–positive cells was significantly greater in the estradiol group than in the placebo group (1 week, 298±24/mm^2 versus 220±9/mm^2, respectively [$P<0.05$]; 4 weeks, 82±8/mm^2 versus 24±7/mm^2, respectively [$P<0.01$]; Figure 4A and 4B). Furthermore, fluorescence immunohistochemistry performed on frozen sections 1 week after MI documented an increase in cells that were double positive for β-galactosidase (indicating BM origin and Tie-2 expression) and the endothelial cell–specific marker isolectin B4 in the estradiol group (Figure 4C). These data indicate that use of estradiol results in an increase in the kinetics of BM-derived EPCs, not only in the circulation but in the myocardium as well, resulting in enhanced incorporation of BM-derived cells into the neovasculature after MI.

eNOS Is Required for Estradiol-Induced Neovascularization

Prior studies have suggested that eNOS is required for estradiol-mediated mobilization of EPCs after arterial injury.[13,19] Figure 5 shows the results of echocardiography before and 1 and 4 weeks after MI in eNOS$^{-/-}$ and WT mice receiving 17β-estradiol pellets. Despite estradiol administration, there was more LV dilation in eNOS$^{-/-}$ mice than in identically treated WT mice, indicating that the beneficial effect of estradiol on LV remodeling after MI was lost in the absence of eNOS (LVDd, 3.9±0.1 versus 4.3±0.1 mm, respectively [$P<0.05$]; LVDs, 2.9±0.2 versus 3.5±0.2 mm, respectively [$P<0.05$]). LV function, assessed by fractional shortening, was also significantly worse despite estradiol treatment in eNOS$^{-/-}$ mice as compared with WT mice. Histological assessment also revealed that eNOS$^{-/-}$ mice showed more LV fibrosis than did WT mice (50.0±2.7% versus 40.1±2.6%, respectively [$P<0.05$]; Figure 5B). Moreover, capillary density 4 weeks after MI was significantly lower in estradiol-treated eNOS$^{-/-}$ mice than in identically

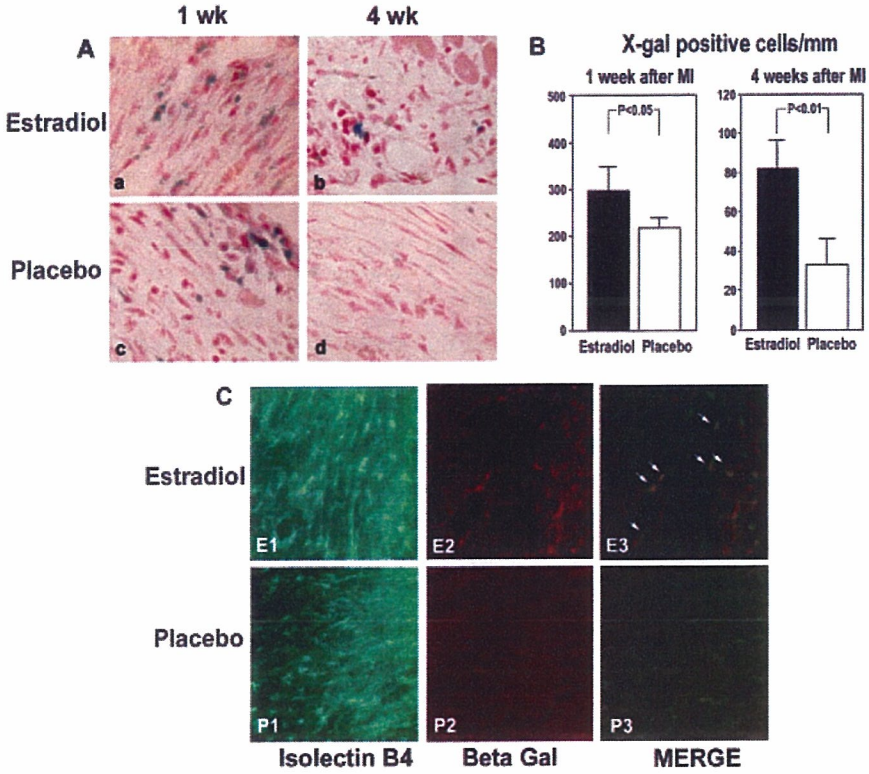

Figure 4. Estradiol enhances the contribution of BM-derived EPCs to myocardial neovascularization. A, Representative photomicrographs after X-gal staining of myocardium in Tie-2/LZ/BMT mice obtained 1 and 4 weeks after MI (a and b are from estradiol group; c and d, from placebo group). X-gal–positive cells (blue) indicate Tie-2–expressing BM-derived EPCs. B, Quantitative analyses of X-gal–positive cells in estradiol and placebo groups 1 and 4 weeks after MI indicate that at both time points after MI, estradiol treatment resulted in greater numbers of Tie-2–expressing cells in the myocardium (n=4 in each group). C, Representative photomicrographs after double-immunofluorescent histochemistry of myocardial tissue in Tie-2/LZ/BMT mice 1 week after MI. Green fluorescence indicates endothelium-specific isolectin B4 binding in estradiol (E1) or placebo (P1) groups; red fluorescence indicates β-gal immunoreactivity in estradiol (E2) or placebo (P2) groups. The double-positive cells (arrows) indicate BM-derived EPCs that have incorporated into the myocardial neovasculature (E3 indicates estradiol; P3, placebo).

treated WT mice (1560±83/mm^2 versus 2335±84/mm^2, respectively [$P<0.01$]; Figure 5C).

EPC counts, documented by FACS analysis and culture assay significantly increased 1 week after MI only in estradiol-treated WT mice as compared with WT mice (culture assay, 588±55/mm^2 versus 284±24 cells/mm^2, respectively [$P<0.01$]; FACS analysis, 13.2±1.1% versus 7.3±0.7%, respectively [$P<0.01$]; Figure 5D). Thus, estradiol failed to increase EPC numbers after MI and failed to improve functional and anatomic preservation after MI in the absence of intact eNOS signaling.

Matrix Metalloproteinase-9 Is Essential for EPC Mobilization Induced by Estradiol After MI

In BM cells of untreated WT mice, mild matrix metalloproteinase-9 (MMP-9) immunoreactivity was noted, whereas it was undetectable in the BM of untreated eNOS$^{-/-}$ mice. In estradiol-treated mice, MMP-9 was strongly induced 1 day after MI in the WT mice, whereas eNOS-null mice revealed a more modest upregulation of MMP-9 expression (Figure 6A). At least a portion of estradiol-induced MMP-9 expression in the BM is from vascular endothelial growth factor (VEGF) receptor 2–expressing cells (Figure 6B) and results in release of soluble kit ligand (Figure 6C), consistent with the documented role of this pathway in vasculogenesis.[20,21]

Next, we assessed MMP-9 activity with the use of gelatin zymography. MMP-9 activity was quantified as a band of gelatinolytic activity, which was based on its specific molecular weight (92 kDa), and was confirmed by comparison with commercially available pure MMP-9 (Figure 7A). As shown in Figure 6, estradiol administration resulted in robust induction of MMP-9 activity in WT (FVB/NJ) mice (for WT mice, 40.0±3.0 pg for estradiol versus 21.0±2.0 pg for placebo). Examination of eNOS-null mice and comparison with WT mice revealed that basal MMP-9 activity was significantly reduced in the eNOS-null mice as compared with WT mice and that estradiol-induced MMP-9 activity was also significantly attenuated in the eNOS-null mice as compared with WT control mice (for eNOS$^{-/-}$ mice, 24.0±6.7 pg for estradiol versus 3.0±0.6 pg for placebo). In direct comparison, there was a significant difference in the induced MMP-9 activity between WT mice (C57BL/6J) and eNOS$^{-/-}$ mice receiving 17β-estradiol pellets (76.3±11.7 versus 24.0±6.7 pg, respectively [$P<0.05$]). These findings suggest that estradiol-induced EPC mobilization after MI might occur by increasing the activity of MMP-9 and that this is, at least in part, dependent on intact eNOS function. This hypothesis was strongly supported by FACS analysis of circulating mononuclear cells from MMP-9$^{-/-}$ mice before and 1 week after MI. Quantification of Sca-1$^+$/Flk-1$^+$ cells revealed that estradiol had no effect on circulating EPC counts after MI in MMP-9–null mice; ie, the MI-induced increase in circulating EPCs was not augmented by estradiol in the absence of MMP-9 (Figure 7B). Most notably, MMP-9–null mice failed to achieve the full benefit of estradiol treatment after MI, showing worse LV dilation and more severely reduced contractile function than was found with WT animals.

Discussion

The present study provides evidence that estradiol mobilizes circulating EPCs from BM, resulting in incorporation into sites of neovascularization in the adult heart after MI. Moreover, this enhanced neovascularization is associated

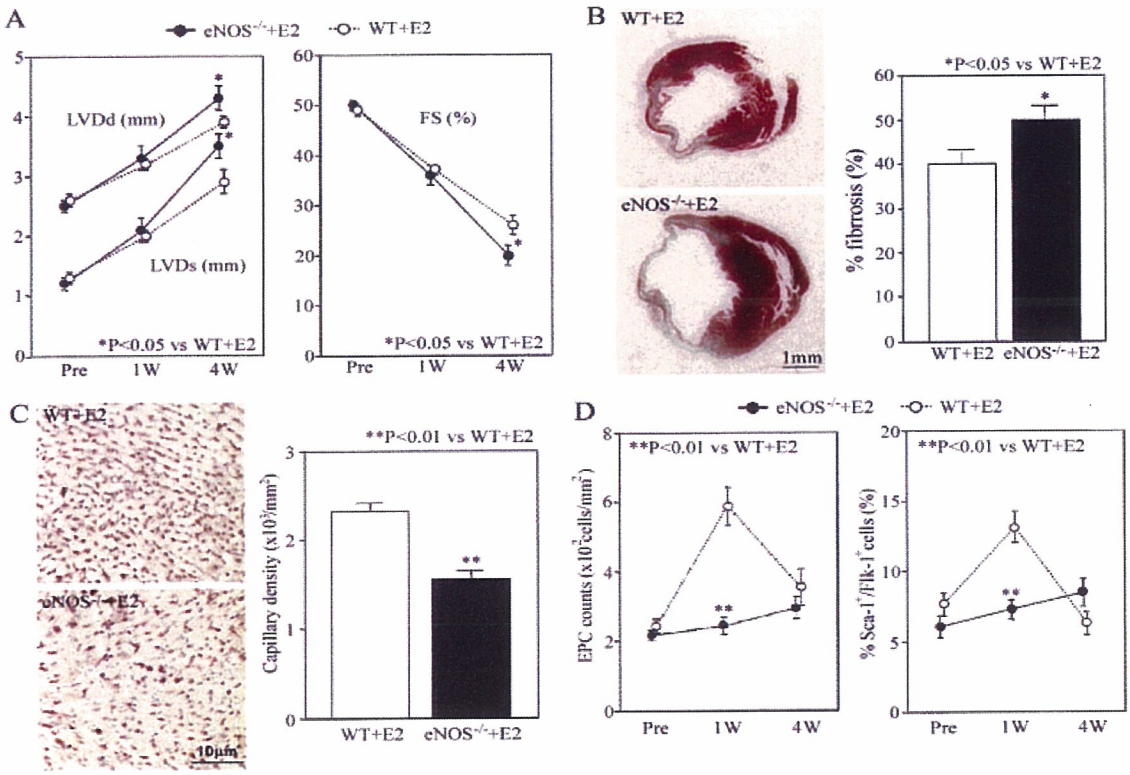

Figure 5. eNOS mediates estradiol-induced improvement in recovery after MI. Ovariectomized estradiol-treated WT and eNOS-null (eNOS$^{-/-}$) mice were subjected to MI and evaluated at serial time points. A, Echocardiography reveals that as compared with identically treated WT mice, eNOS-deficient mice develop worse LV dilation and contractile dysfunction despite estradiol treatment (n=5 in each group). B, Elastic-trichrome staining reveals that as compared with similarly treated WT mice, estradiol-treated eNOS-deficient mice have more extensive LV fibrosis after MI (n=5 in each group). C, Capillary density, assessed by isolectin B4 immunohistochemistry, is decreased in estradiol-treated eNOS-null mice as compared with WT mice (n=5 in each group). D, The increase in circulating EPCs seen in estradiol-treated WT mice after MI, assessed by culture assay (left) and FACS analysis (right) for Sca-1$^+$/Flk-1$^+$ cells, is attenuated in eNOS$^{-/-}$ mice (n=4 or 5 in each group at each time point).

with a reduction in the extent of LV scarring and with enhanced preservation of LV function in the chronic phase after MI. Most notably, this salutary effect of estradiol appears to be dependent on the ability of estradiol to modulate the kinetics of BM-derived progenitor cells. This latter phenomenon is supported by the fact that eNOS– and MMP-9–null mice fail to mobilize EPCs in response to estradiol administration and also shows significantly worse functional outcome, despite estradiol treatment.

There are now abundant data documenting a role for EPCs in vascular biology. Animal data indicate that BM-derived cells are a part of normal blood vessel homeostasis and can be recruited by a variety of stimuli, including ischemia, tissue injury, and cytokines.[8–10,22,23] In humans, the participation of EPCs in vessel formation has been documented in elegant studies in sex-mismatched transplant recipients in which donor cells have been identified in the vasculature of recipient organs.[24–26] Most recently, the potential for these cells to induce neovascularization has been exploited in therapeutic trials.[27,28]

In the context of these discoveries, in humans, observational data linking the quantity and phenotype of circulating progenitor cells to various vascular processes have begun to accumulate. Vasa et al[28a] first noted a statistical relationship between circulating progenitor cell numbers and phenotype and the presence of various traditional risk factors for coronary artery disease. Others have since corroborated and extended these findings,[29] implying that the "health" of the underlying pool of progenitor cells might itself be a major determinant of the overall risk for vascular disease.[18] Conversely, EPCs have also been identified as participants in tumor vascularization[30,31] and, as such, as possible targets for antitumor therapies.[32–34]

Juxtaposed against these findings is the current paradox revolving around the role of sex hormones, and in particular estrogen, in cardiovascular health and disease. Animal data and extensive observational human studies have implied a protective role of estradiol against cardiovascular disease.[35] Randomized clinical trials, however, have not yielded the anticipated results.[36] The reasons for the failure of estrogen replacement to reduce cardiovascular mortality remain to be clarified[37]; however, our data, along with the body of evidence implicating EPCs as major factors in vascular processes, illuminate another possibility.

The present findings suggest that in the presence of estradiol, the reparative process normally invoked by myocardial injury is markedly enhanced. The enhanced repair process is documented anatomically and functionally and, most notably, appears to be significantly derived via the effect of estradiol on EPC mobilization and recruitment. This

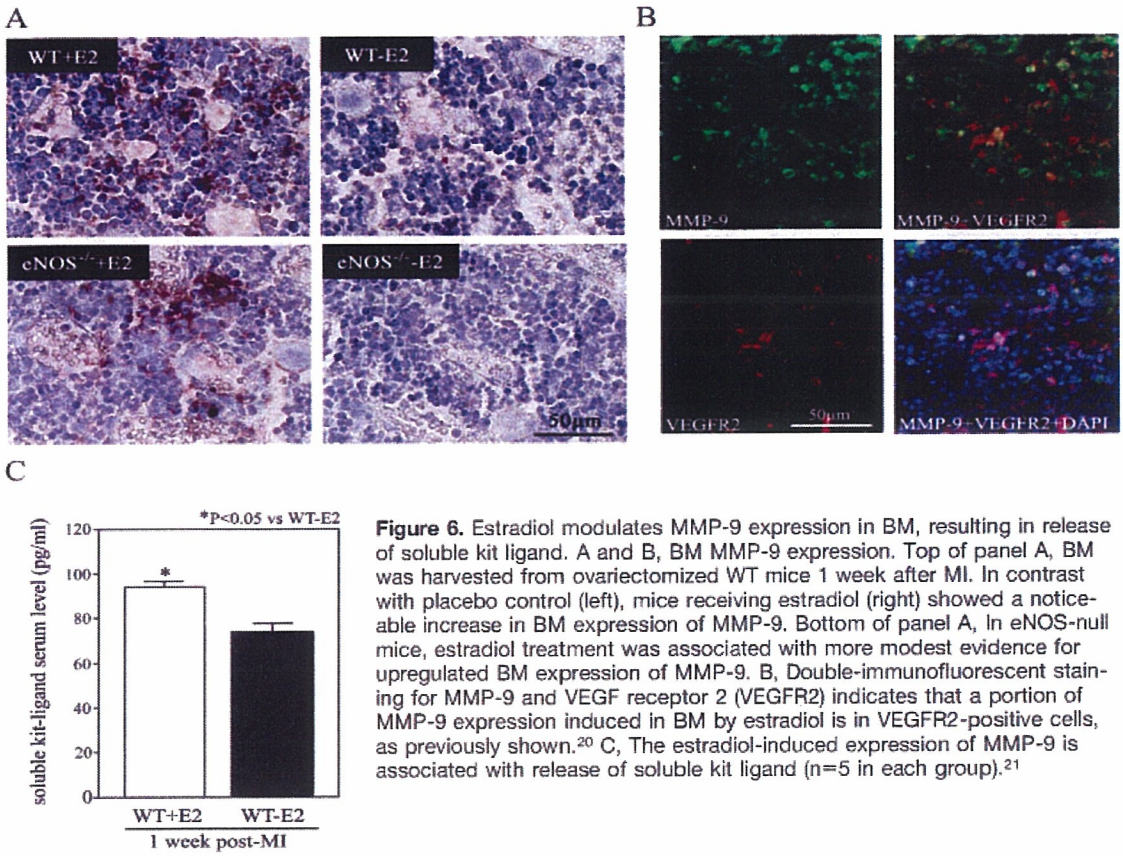

Figure 6. Estradiol modulates MMP-9 expression in BM, resulting in release of soluble kit ligand. A and B, BM MMP-9 expression. Top of panel A, BM was harvested from ovariectomized WT mice 1 week after MI. In contrast with placebo control (left), mice receiving estradiol (right) showed a noticeable increase in BM expression of MMP-9. Bottom of panel A, In eNOS-null mice, estradiol treatment was associated with more modest evidence for upregulated BM expression of MMP-9. B, Double-immunofluorescent staining for MMP-9 and VEGF receptor 2 (VEGFR2) indicates that a portion of MMP-9 expression induced in BM by estradiol is in VEGFR2-positive cells, as previously shown.[20] C, The estradiol-induced expression of MMP-9 is associated with release of soluble kit ligand (n=5 in each group).[21]

latter conclusion is supported by the fact that the therapeutic effect of estradiol has been shown to be reduced in both eNOS-null and MMP-9–null mice, which both showed reduced or absent estradiol-induced EPC mobilization.

VEGF and granulocyte-macrophage colony–stimulating factor can mobilize EPCs from BM into the peripheral circulation, and they have also been shown to enhance recovery after ischemic injury.[38,39] Although VEGF-A was initially considered to promote neovascularization through mitogenic and promigratory effects on fully differentiated endothelial cells, it is now clear that VEGF-A also acts to mobilize BM-derived EPCs, resulting in contribution to postnatal neovascularization via vasculogenesis.

The requirement for eNOS for EPC release from the BM has been noted previously,[19] as has its role in mediating a variety of the effects of estradiol on the vasculature.[13,40–47] The present study advances the notion that the cardioprotective effect of estradiol is mediated, at least in part, by its ability to augment the mobilization and recruitment of BM progenitors for vascular repair. This concept may offer important clues about the failure of hormone replacement in clinical trials. Moreover, because the eNOS-null mouse used in our studies has been shown to be deficient in mobilizing EPCs under multiple stimuli,[19] the specific mechanism involved in estradiol-mediated EPC release remains to be ascertained.

Our previous findings[11] have also revealed that estradiol administration induces the recruitment and incorporation of EPCs into the endometrial neovasculature in ovariectomized mice. The present study reveals that estradiol can augment EPC mobilization not only into physiological (eg, reproductive) neovascularization but also into sites of neovascularization in adult organs under pathologic conditions. In addition to the obvious application of these findings to the understanding of sex differences in recovery from MI, they may also have important implications for understanding the pathophysiology of certain hormone-dependent solid tumors that have also been shown to be angiogenesis dependent.[48]

In the present study, the beneficial effects of estradiol were not observed in the acute phase after MI, which is consistent with prior reports.[49] These findings may provide clues toward understanding the seemingly conflicting experimental, epidemiological, and clinical data on sex differences in outcome after MI.[50,51] On one hand, female sex and intact ovarian function are associated with a lower risk of death from MI before menopause, whereas in postmenopausal life, female sex has been consistently associated with a worse prognosis after MI. Could EPC function be a factor to explain this apparent discrepancy? In this context, it is noteworthy that stem cell mobilization is markedly attenuated in older patients[52] and that EPC numbers have also been shown to be reduced with age. We speculate that, among other reasons, the failure of postmenopausal estrogen replacement could be linked to the decreased availability of EPCs, thereby obviating some of the potential benefits of estradiol while still exposing patients to the risks of increased thrombosis, among others.[53] It is further tempting to speculate about a potential role for EPCs in the protection against MI that has been

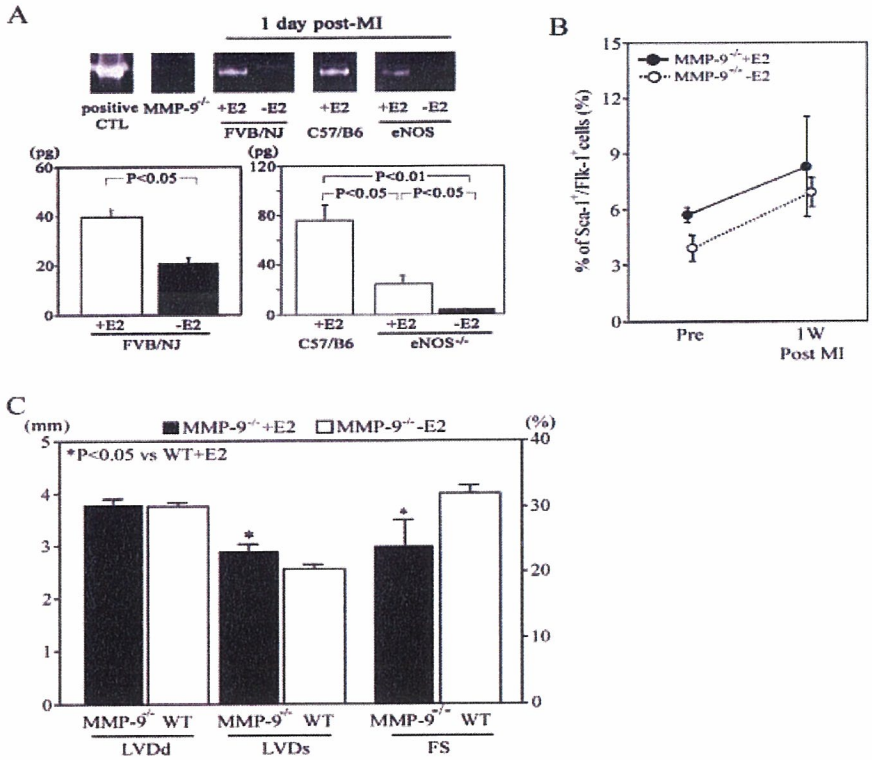

Figure 7. MMP-9 expression is critical for estradiol-mediated mobilization of BM-derived progenitor cells and enhanced functional recovery after MI. A, Gelatin zymography reveals estradiol-induced MMP-9 activity that is attenuated in the absence of eNOS. Spleens were harvested from WT (FVB/NJ and C57/B6) and eNOS-deficient mice 1 day after MI and assessed for MMP-9 activity. Top of panel A, Zymograms show increased lytic activity in estradiol-treated as compared with control mice (CTL), with an apparent decrease in estradiol-induced MMP-9 activity in eNOS-null mice. Bottom of panel A, Net MMP-9 activity is quantified and shown to be upregulated by estradiol in WT mice. In eNOS-deficient mice, however, MMP-9 activity is markedly reduced under basal and estradiol-stimulated conditions (n=5 in each group). B, Estradiol-induced EPC mobilization is absent in MMP-9–null mice. MI was induced in ovariectomized estradiol- or placebo-treated MMP-9$^{-/-}$ mice, and FACS analysis for Sca-1/Flk-1 was performed on circulating blood mononuclear cells harvested 1 week after MI. As compared with placebo, estradiol administration resulted in no significant increase in circulating EPC numbers, indicating that the augmentation of EPC kinetics in the setting of MI is MMP-9 dependent (n=5 in each group). C, Ovariectomized estradiol-treated WT and MMP-9–null mice were subjected to MI and evaluated by echocardiography 4 weeks later, revealing that MMP-9–deficient mice develop worse LV dilation and contractile dysfunction despite estradiol treatment than do identically treated WT mice (n=5 in each group).

documented in premenopausal females and the markedly accelerated incidence of cardiac events that occurs after menopause. Estradiol mobilizes EPCs on a cyclic basis, in part (apparently) for uterine vascularization. However, a side effect of the increased circulating supply of these cells might be the incidental repair of sites of vascular injury, thereby deterring the progression of atherosclerosis.[54] If it is assumed that the population of BM cells capable of differentiation into EPCs is finite, it is possible that the monthly consumption of these progenitors for uterine vascularization during premenopause results in depletion of these cells over time, so that after menopause the availability of EPCs is relatively deficient in females as compared with males.

In the present study, estradiol attenuated the impairment of LV function in the chronic phase after MI. The data provide clear evidence that this is due, at least in part, to an estradiol-mediated increase in incorporation of BM-derived EPCs into sites of neovascularization. However, these findings do not exclude other potential mechanisms whereby estradiol may inhibit the adverse LV remodeling after MI and subsequent congestive heart failure, such as inhibition of angiotensin-converting enzyme activity and regulation of the endothelin system.[55] Further investigation will clarify these mechanisms and their relative contributions to post-MI recovery.

The results of these experiments may have clinical relevance from 2 perspectives. First, and most direct, there are implications in the ongoing debate about hormone replacement therapy in postmenopausal women, inasmuch as our results could suggest a potential benefit for the management of MI in postmenopausal women, preventing LV dysfunction in the chronic phase after MI. Second, and more comprehensive, the present study has implications for our general understanding of recovery from MI, suggesting that enhanced recruitment and incorporation of BM-derived EPCs may play a significant role in restoring myocardial perfusion, salvaging jeopardized tissue, and attenuating late-phase remodeling and loss of function after MI.

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Disclosures

None.

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CLINICAL PERSPECTIVE

From a clinical perspective, the findings in the present study may have several implications. Most general, but perhaps most important, these findings provide another piece of evidence indicating that preservation of the myocardial microvasculature in the peri-infarct zone is associated with preservation of overall left ventricular function. These data also indicate that mobilizing endothelial progenitor cells and enhancing the contribution of these bone marrow–derived stem cells into the myocardial microvasculature can achieve improved outcome after myocardial infarction. Finally, the mechanisms involving endothelial nitric oxide synthase signaling and matrix metalloproteinase-9 that are revealed may provide suitable therapeutic targets for human study.

Dose-Dependent Contribution of CD34-Positive Cell Transplantation to Concurrent Vasculogenesis and Cardiomyogenesis for Functional Regenerative Recovery After Myocardial Infarction

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Background—Multilineage developmental capacity of the $CD34^+$ cells, especially into cardiomyocytes and smooth muscle cells (SMCs), is still controversial. In the present study we performed a series of experiments to prove our hypothesis that vasculogenesis and cardiomyogenesis after myocardial infarction (MI) may be dose-dependently enhanced after $CD34^+$ cell transplantation.

Methods and Results—Peripheral blood $CD34^+$ cells were isolated from total mononuclear cells of patients with limb ischemia by apheresis after 5-day administration of granulocyte colony-stimulating factor. PBS and 1×10^3 (low), 1×10^5 (mid), or 5×10^5 (high) $CD34^+$ cells were intramyocardially transplanted after ligation of the left anterior descending coronary artery of nude rats. Functional assessments with the use of echocardiography and a microtip conductance catheter at day 28 revealed dose-dependent preservation of left ventricular function by $CD34^+$ cell transplantation. Necropsy examination disclosed dose-dependent augmentation of capillary density and dose-dependent inhibition of left ventricular fibrosis. Immunohistochemistry for human-specific brain natriuretic peptide demonstrated that human cardiomyocytes were dose-dependently observed in ischemic myocardium at day 28 (high, 2480±149; mid, 1860±141; low, 423±9; PBS, 0±0/mm^2; $P<0.05$ for high versus mid and mid versus low). Immunostaining for smooth muscle actin and human leukocyte antigen or *Ulex europaeus* lectin type 1 also revealed dose-dependent vasculogenesis by endothelial cell and SMC development after $CD34^+$ cell transplantation. Reverse transcriptase–polymerase chain reaction indicated that human-specific gene expression of cardiomyocyte (brain natriuretic peptide, cardiac troponin-I, myosin heavy chain, and Nkx 2.5), SMC (smooth muscle actin and sm22α), and endothelial cell (CD31 and KDR) markers were dose-dependently augmented in MI tissue.

Conclusions—Human $CD34^+$ cell transplantation may have significant and dose-dependent potential for vasculogenesis and cardiomyogenesis with functional recovery from MI. **(*Circulation*. 2006;113:1311-1325.)**

Key Words: angiogenesis ■ cell therapy ■ myocardial infarction ■ transplantation ■ vasculogenesis

The various cell types that make up the blood are of mesodermal origin and emanate from a common pool of hematopoietic stem cells (HSCs). During embryogenesis, hematopoietic and endothelial lineage cells[1,2] are derived from common progenitor cells (hemangioblasts).[3–6] In adults, $CD34^+$ cells had been considered to be HSCs and clinically applied to the field of hematology for HSC transplantation.[7,8] Recently, human peripheral blood $CD34^+$ cells were reported to be an endothelial progenitor cell (EPC)–enriched population as well as a HSC fraction.[9] Thereafter, therapeutic application of $CD34^+$ cells for vascular regeneration has been performed in many preclinical studies. In the case of immunodeficient rats with acute myocardial infarction (MI), transplanted human $CD34^+$ cells or ex vivo expanded EPCs incorporated into the site of myocardial neovascularization, differentiated into mature endothelial cells (ECs) (vasculo-

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genesis), augmented capillary density, inhibited myocardial fibrosis and apoptosis, and preserved left ventricular (LV) function.[10–12] In our institution, a phase I/II clinical trial of $CD34^+$ cell transplantation has already been started in patients with chronic critical limb ischemia.

Recently, Orlic et al[13] and Jackson et al[14] reported translineage differentiation of adult mouse HSCs into cardiomyocytes when introduced into heart by intramyocardial injection or via the circulation. This is followed by demonstrating that mouse c-Kit–positive bone marrow cells differentiate into myocardium and blood vessels in vivo.[15] Further reports[16,17] presented development of human peripheral blood $CD34^+$ cells into cardiomyocytes, smooth muscle cells (SMCs), and ECs in a mouse model of acute MI. On the other hand, other investigators indicated that transplanted HSCs were unable to transdifferentiate into cardiomyocytes after MI.[18,19] Given these extensive and controversial investigations into the potency of so-called stem/progenitor cells derived from peripheral blood and bone marrow in experimental models, we conducted this study to investigate not only vasculogenesis but also cardiomyogenesis derived from human $CD34^+$ cells, the most practical human cells for clinical medicine, in a dose-dependent manner.

The present results of human CD34-positive cell transplantation into an immunodeficient rat MI model demonstrated that the collaborative multilineage differentiation potential of $CD34^+$ cells into not only ECs but cardiomyocytes and SMCs was enhanced by cell dose escalation and was conducive to heart regeneration in terms of functional and histological recovery through vasculogenesis and cardiomyogenesis.

Methods

Isolation of $CD34^+$ Cells From Patients With Critical Limb Ischemia

Peripheral blood total mononuclear cells were obtained from 2 male patients aged 21 and 40 years with Buerger disease by apheresis after 5-day subcutaneous administration of granulocyte colony-stimulating factor (G-CSF) (10 μg/kg per day). Bone marrow–derived $CD34^+$ cells were isolated from total mononuclear cells by the magnetic cell sorting system CliniMACS (Miltenyi Biotec Inc, Auburn, Calif).[20] These patients received intramuscular transplantation of 10^5 $CD34^+$ cells/kg according to the protocol of a phase I/II dose-escalation clinical trial. The $CD34^+$ cell fraction had a purity of >99%, as determined by fluorescence-activated cell sorting (FACS) analysis with the use of a CD34-specific monoclonal antibody (Becton Dickinson, San Jose, Calif) (Figure 1a and 1b). Remaining $CD34^+$ cells were used for the following experiments. Informed consent with regard to the cell therapy and experimental use of the remaining cells was obtained from the patients before the case registration. The clinical study protocol was approved by the institutional ethics committees of Kobe Institute of Biomedical Research and Innovation and Kobe City General Hospital.

Animals

Female athymic nude rats (F344/N Jcl rnu/rnu, CIEA Japan, Inc, Tokyo, Japan) aged 7 to 8 weeks and weighing 130 to 145 g were used in this study. The institutional animal care and use committees of RIKEN Center for Developmental Biology approved all animal procedures, including human cell transplantation.

Induction of MI and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 and 10 mg/kg IP, respectively). MI was induced by ligating the left anterior descending coronary artery as described previously.[12] In brief, after the fourth to fifth intercostal space was opened, the heart was exteriorized, and the pericardium was incised. Thereafter, the heart was held with forceps, and MI was induced by ligating the left anterior descending coronary artery near its origin with a 6-0 Proline suture. Twenty minutes after MI, rats received intramyocardial transplantation of 1×10^5 (low group), 1×10^5 (mid group), or 5×10^5 (high group) $CD34^+$ cells resuspended with 120 μL of PBS or the same volume of PBS without cells (n=12 in each group when the first patient's cells were used; n=4 in each group for the second patient's cells). After the injection was completed, the thorax was closed.

Flow Cytometry Studies and Monoclonal Antibodies

Regular flow cytometric profiles were analyzed with a FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, Calif). The instrument was aligned and calibrated daily with the use of a 4-color mixture of CaliBRITE beads (BD Biosciences, San Jose, Calif) with FACSComp software (BD Biosciences). Dead cells were excluded from the plots on the basis of propidium iodide (PI) staining (Sigma Co, St Louis, Mo). $CD34^+$ cells were washed twice with Hanks' balanced salt solution (HBSS) containing 3.0% heat-activated fetal calf serum (FCS), incubated with 10 μL of FcR blocking regent to increase the specificity of monoclonal antibodies (Milteyi Biotic) for 20 minutes at 4°C, and incubated with the monoclonal antibodies for 30 minutes at 4°C. The stained cells were washed 3 times with PBS containing 3.0% FCS, resuspended in 0.5 mL of HBSS/3% FCS/PI, and analyzed by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The following monoclonal antibodies were used to characterize the $CD34^+$ cell population: CD34-FITC (clone My10, BD), CD34-PE (clone 581, Pharmingen, San Diego, Calif), CD45-FITC (clone HI30, Pharmingen), CD31-FITC (clone WM59, Pharmingen), KDR (Sigma), AC133-APC (clone AC133, Pharmingen), VE-cadherin-APC (HyCult Biotechnology, Uden, The Netherlands), IgG1-FITC isotype controls (Pharmingen), IgG1-PE isotype controls (Pharmingen), IgG1-APC isotype controls (Pharmingen), IgG2a-APC isotype controls (Pharmingen), and PI (Sigma Chemical Co, St Louis, Mo).

Physiological Assessment of LV Function Using Echocardiography and Microtip Conductance Catheter

Transthoracic echocardiography (SONOS 5500, Philips Medical Systems) was performed to evaluate LV function immediately before and 5 and 28 days after MI. Under general anesthesia with ketamine and xylazine, LV end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) and fractional shortening (FS) were measured at the midpapillary muscle level. Regional wall motion score (RWMS) was evaluated per published criteria.[21] All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function.[22] A 2.0F micromanometer-tipped conductance catheter (SPR 838, Millar Instruments Inc, Houston, Tex) was inserted via the right carotid artery into the LV cavity. LV pressure and its derivative (LV dP/dt) were continuously monitored with a multiple recording system (Pressure-Volume Conductance System ARIA and Pressure-Volume Analysis Using P-V Analysis Software [Millar Instruments Inc, Houston, Tex] and Power Laboratory DAQ System [ADInstrument, Australia]).

Heart rate, LV end-diastolic pressure (LVEDP), LV ejection fraction (EF), and the maximum and minimum LV dP/dt (+dP/dt and −dP/dt, respectively) were recorded continuously for 20 minutes. All data were acquired under stable hemodynamic conditions. All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

Tissue Harvesting

All rats were killed 28 days after transplantation with an overdose of ketamine and xylazine. At necropsy, hearts were sliced in a broadleaf

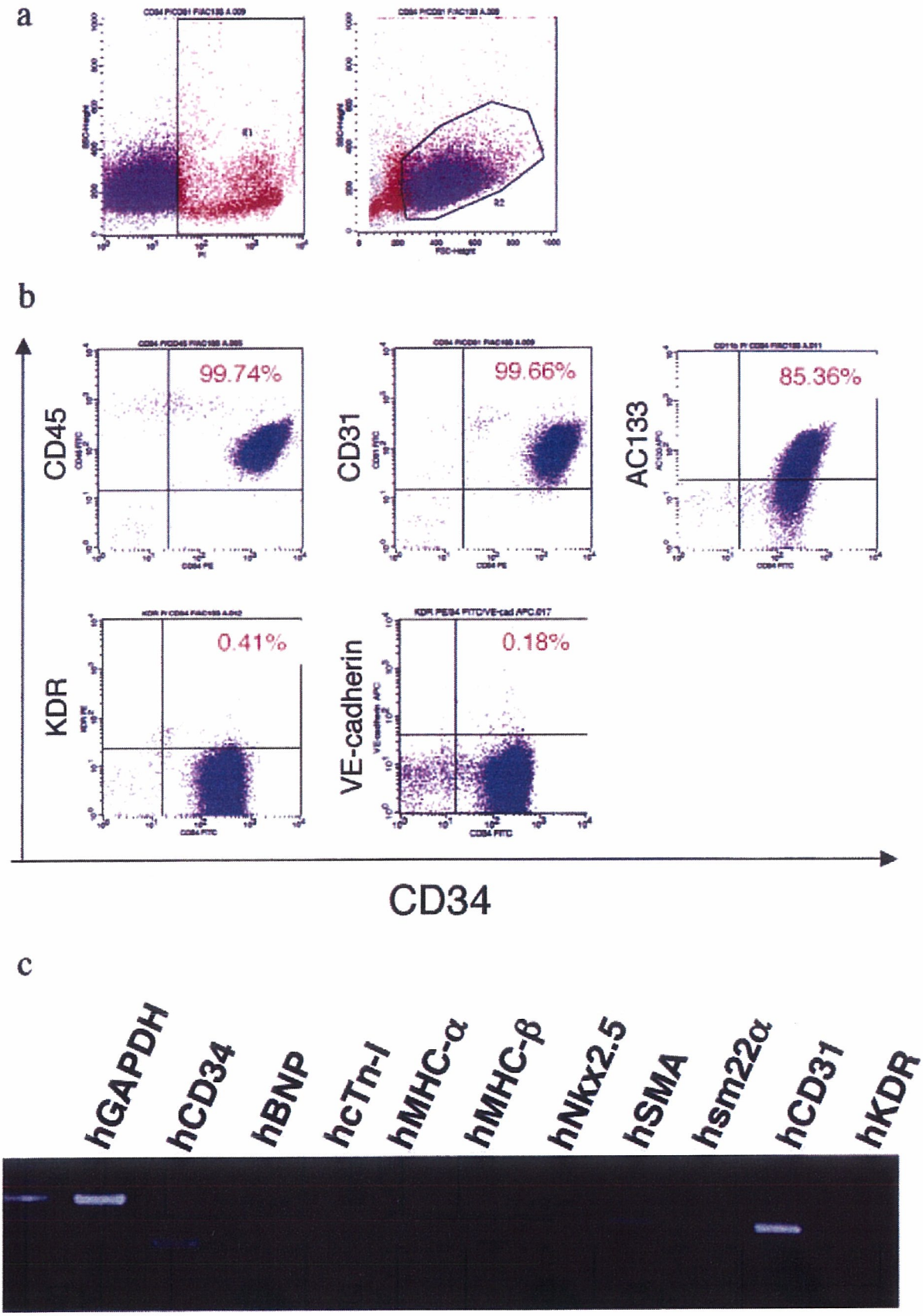

Figure 1. Representative FACS profile and RT-PCR of CD34+ cells isolated from a critically ischemic patient by magnetic cell sorting system. a, Dead cells were excluded by PI staining, and then mononuclear cell (MNC) population within live cells was assessed. b, The cells were stained with CD45, CD31, AC133, KDR, VE-cadherin, and CD34. Numbers are percentage of double-positive cells in each staining. c, RT-PCR analysis for human-specific genes of cardiomyocyte, SMC, and EC lineages in freshly isolated CD34+ cells.

fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −80°C for Masson trichrome staining, immunohistochemistry, and fluorescence in situ hybridization (FISH). Rat hearts in OCT blocks were sectioned, and 5-μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 minutes and stained immediately. Total RNA was isolated by selective dissection of peri-infarct area in LV myocardium for reverse transcriptase–polymerase chain reaction (RT-PCR).

Morphometric Evaluation of Capillary Density and Infarct Size

Histochemical staining with isolectin B4 (Vector Laboratories, Burlingame, Calif) was performed, and capillary density was morphometrically evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded left anterior descending coronary artery. Capillaries were recognized as tubular structures positive for isolectin B4. To elucidate the severity of myocardial fibrosis, Masson trichrome staining was performed on frozen sections from each tissue block, and the stained sections were used to measure the average ratio of fibrosis area to entire LV area (percent fibrosis area). All morphometric studies were performed by 2 examiners who were blinded to treatment.

Immunofluorescence Staining

To detect transplanted human cells in rat ischemic myocardium, immunohistochemistry was performed with following human-specific antibodies: human leukocyte antigen (HLA)–ABC (BD Pharmingen) and human nuclei antibody (HNA) (Chemicon International, Inc, Temecula, Calif) to detect various kinds of human cells, human-specific brain natriuretic peptide (hBNP),[23] which was kindly given by Dr Hiroshi Itoh of Kyoto University Kyoto, Japan, to detect human cardiomyocytes, and human-specific *Ulex europaeus* lectin type 1 (UEA-1) (Vector Laboratories, Inc)[24] for human ECs. Staining specificity of hBNP, HLA-ABC, HNA, and UEA-1 against human cells and lack of cross-reactivity to rat cells were confirmed by histochemical staining with the use of human and rat heart samples (data not shown). Double immunohistochemistry with HNA and cardiac troponin I (cTn-1) (Chemicon International, Inc) was performed to detect double-positive cells as human cardiomyocytes in rat myocardium. Double immunohistochemistry with HLA-ABC and smooth muscle actin (SMA) was performed to detect double-positive cells as human SMCs in rat myocardium. Similarly, double immunohistochemistry with hUEA-1 and SMA and for von Willebrand factor (vWF) (Chemicon International, Inc) and HNA was performed to detect UEA-1–positive cells and double-positive cells of vWF and HNA as human ECs in ischemic myocardium. The secondary antibodies for each immunostaining are as follows: FITC goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, Me) for hBNP staining, Alexa Flour 594–conjugated goat anti-mouse IgG_1 (Molecular Probes) for HLA-ABC staining, Alexa Flour 488–conjugated goat anti-mouse IgG_{2a} (Molecular Probes, Carlsbad, Calif) for cTn-I staining, Alexa Flour 488–conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for SMA, Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pa) for hUEA-1, Alexa Flour 488– and 594–conjugated goat anti-mouse IgG1 for HNA, and Alexa Flour 594–conjugated goat anti-rabbit IgG for vWF. DAPI solution was applied for 5 minutes for nuclear staining. Number of human cardiomyocytes, total (both human and rat) cardiomyocytes in ischemic myocardium detected as hBNP-positive cells, number of human SMCs as double-positive cells for HLA and SMA, and number of human ECs as hUEA-1–positive cells 28 days after MI were morphometrically quantified with the use of 5 randomly selected fields (from peri-infarction area to fibrosis area) of tissue sections.

Fluorescence In Situ Hybridization

To identify whether cardiac repair occurred through cell fusion in rat ischemic myocardium, FISH was performed with human Y chromosome painting probe and rat genome probe (nick translation methods) in MI tissue. Tissue sections were fixed immediately with 4.0% paraformaldehyde at 4°C for 20 minutes and predenatured, dehydrated, and denatured according to the manufacturer's protocol. Sections were hybridized with a Cy-3–conjugated DNA probe for human Y chromosomes and a biotin-conjugated probe for rat genome overnight at 37°C. After posthybridization wash, TexRed-conjugated streptavidin was applied, and slides were counterstained with DAPI and examined.

RT-PCR Analysis of $CD34^+$ Cells and Ischemic Heart Tissue

Total RNA was obtained from freshly isolated peripheral blood $CD34^+$ cells of a patient and tissues of rat ischemic LV at day 28 with the use of TRIzol (Life Technologies, St Paul, Minn) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the use of the RNA LA PCR Kit version 1.1 (Takara, Otsu, Japan), amplified by *Taq* DNA polymerase (Advantage-GC cDNA PCR Kit, Clontech and AmpliTaq Gold DNA polymerase, Applied Biosystems). PCR was performed with a PCR thermocycler (MJ Research PTC-225). The human GAPDH (hGAPDH), total (human and rat) GAPDH, human CD34 (hCD34), hBNP, human cardiac troponin-I (hcTn-I), human myosin heavy chain-β (hMHC-β), human KDR (hKDR), and human Nkx 2.5 (hNkx 2.5) were amplified by *Taq* DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems) under the following conditions: 35 cycles of 30 seconds of initial denaturation at 94°C, annealing at 56°C for 1 minute, and 30 seconds of extension at 72°C according to the manufacturer's instructions. Human myosin heavy chain-α (hMHC-α), human SMA (hSMA), human sm22α (hsm22α), and human CD31 (hCD31) were amplified by *Taq* DNA polymerase (Advantage-GC cDNA PCR Kit, Clontech, Mountain View, Calif) under the following conditions: 37 cycles of 30 seconds of initial denaturation at 94°C, annealing at 68°C for 3 minutes, and 7 minutes of elongation at 64°C according to the manufacturer's instructions. Subsequently, PCR products were visualized in 1.5% ethidium bromide–stained agarose gels. Human heart RNA distributed from Clontech (premium RNA) was used as positive control. To quantify human-specific cardiomyogenic and vasculogenic gene expression in rat ischemic myocardium after human $CD34^+$ cell transplantation, we measured the band intensity of the RT-PCR image. Each gel was photographed onto positive/negative Polaroid film under ultraviolet illumination. The negative images were then captured with the use of an image scanner. After the images were recorded in a computer, the band intensities were processed with the NIH Image program (version 1.62) as described previously.[25] These band intensities were used to calculate the ratio of human-specific marker (hGAPDH, hBNP, hcTn-I, hMHC-α, hMHC-β, hNkx 2.5, hSMA, hsm22α, hKDR, and hCD31) expression to total GAPDH expression.

Primers

To avoid interspecies cross-reactivity of the primer pairs between human and rat genes, we designed the following human-specific primers using Oligo software (Takara). All primer pairs did not show cross-reactivity to rat genes (data not shown). Primer pairs were as follows: hBNP primer sequence (146 bp): sense GCA AAA TGG TCC TCT ACA CC; antisense CAG GAC TTC CTC TTA ATG CC; hcTn-I primer sequence (218 bp): sense AAT TGC AGC TGA AGA CTC TG; antisense GAC TTT TGC CTC TAT GTC GT; hMHC-β primer sequence (214 bp): sense GCT AAA GGT CAA GGC CTA CA; antisense GCA GAT CAA GAT CTG GCA AA; hNkx 2.5 primer sequence (205 bp): sense GAG AGT TTG TGG CGG CGA TT; antisense CGA CGG CGA GAT AGC AAA GG; hMHC-α primer sequence (413 bp): sense GTC ATT GCT GAA ACC GAG AAT G; antisense GCA AAG TAC TGG ATG ACA CGC T; hSMA primer sequence (485 bp): sense TCT GGA CGC ACA ACT GGC ATC GT; antisense TAC ATA GTG GTG CCC CCT GAT AG; hsm22-α primer sequence (468 bp): sense CGG CTG GTG GAG TGG ATC ATA; antisense CCC TCT GTT GCT GCC CAT CTG A; hCD31 primer sequence (469 bp): sense AGG TCA AGC AGC ATC GTG GTC AAC AT; antisense TTG TCT TTG AAT ACC GCA G; hCD34 primer sequence (380 bp): sense AAT GAG GCC ACA ACA