Therapeutic angiogenesis by ex vivo expanded erythroid progenitor cells

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Submitted 31 March 2006; accepted in final form 17 September 2006

Sasaki S, Inoguchi T, Muta K, Abe Y, Zhang M, Hiasa K, Egashira K, Sonoda N, Kobayashi K, Takayanagi R, Nawata H. Therapeutic angiogenesis by ex vivo expanded erythroid progenitor cells. Am J Physiol Heart Circ Physiol 292: H657–H665, 2007. First published September 22, 2006; doi:10.1152/ajpheart.00343.2006.—Recent reports have demonstrated that erythroid progenitor cells contain and secrete various angiogenic cytokines. Here, the impact of erythroid colony-forming cell (ECFC) implantation on therapeutic angiogenesis was investigated in murine models of hindlimb ischemia. During the in vitro differentiation, vascular endothelial growth factor (VEGF) secretion by ECFCs was observed from day 3 (burst-forming unit erythroid cells) to day 10 (erythroblasts). ECFCs from day 5 to day 7 (colony-forming unit erythroid cells) showed the highest VEGF productivity, and day 6 ECFCs were used for the experiments. ECFCs contained larger amounts of VEGF and fibroblast growth factor-2 (FGF-2) than peripheral blood mononuclear cells (PBMNCs). In tubule formation assays with human umbilical vein endothelial cells, ECFCs stimulated 1.5-fold more capillary growth than PBMNCs, and this effect was suppressed by antibodies against VEGF and FGF-2. Using an immunodeficient hindlimb ischemia model and laser-Doppler imaging, we evaluated the limb salvage rate and blood perfusion after intramuscular implantation of ECFCs. ECFC implantation increased both the salvage rate (38% vs. 0%, P < 0.05) and the blood perfusion (82.8% vs. 65.6%, P < 0.01). In addition, ECFCs implantation also significantly increased capillaries with recruitment of vascular smooth muscle cells and the capillary density was 1.6-fold higher than in the control group. Continuous production of human VEGF from ECFCs in the skeletal muscle was confirmed at least 7 days after the implantation. Implantation of ECFCs promoted angiogenesis in ischemic limbs by supplying angiogenic cytokines (VEGF and FGF-2), suggesting a possible novel strategy for therapeutic angiogenesis.

peripheral arterial disease; transplantation; ischemia

ALTERNATIVE THERAPIES for clinical limb ischemia have become very important for patients with peripheral arterial disease (PAD), who cannot undergo surgical or percutaneous revascularization. Pharmacological treatments have been shown to have no favorable effects on the natural history of critical limb ischemia (14). Delivery of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (13, 35, 38), fibroblast growth factor-2 (FGF-2) (3, 27), hepatocyte growth factor (22, 23), stromal cell-derived factor-1α (10, 39), and placental growth factor (18), using recombinant proteins or gene transfer has been considered for alternative treatment of PAD, and their efficacy has been demonstrated. However, angiogenesis is a well-harmonized process established by vascular network maturation and remodeling and involves the recruitment of mural cells (pericytes and smooth muscle cells) to the nascent endothelium. This process may be too complicated for effective stimulation by administration of a single angiogenic factor.

Endothelial progenitor cells (EPCs) have been shown to participate in postnatal neovascularization after mobilization from the bone marrow (1). Therapeutic induction of EPCs obtained from ex vivo expansion of peripheral blood (15), cord blood (24), or bone marrow (34) improved blood perfusion after ischemia and rescued ischemic limbs from autoamputation in animal models, although preparation of the large numbers of EPCs required for a therapeutic effect is difficult. Bone marrow mononuclear cells (BMMNCs) contain not only EPCs but also various potent angiogenic cytokines (16), and cell therapy for PAD using BMMNCs produced feasible angiogenic effects in experimental limb ischemia and clinical trials (36). On the other hand, implantation of peripheral blood mononuclear cells (PBMCNs) also showed effective induction of angiogenesis, although the implanted PBMCNs contained considerably fewer CD34-positive (CD34+) cells than the BMMNCs (0.02% vs. 2.4%) (11). These results may provide the concept that the effect of PBMCNs or BMMNCs is mainly derived from the supply of angiogenic factors rather than the involvement of EPCs.

Recent reports have demonstrated that burst-forming unit erythroid (BFU-E) progenitor cells express high levels of VEGF mRNA (19, 29) and that erythroblasts secrete VEGF and placental growth factor proteins during in vitro differentiation (37). Although these results suggest an important role for erythroid progenitor cells in angiogenesis, no studies have reported in vivo evidence of angiogenesis induction by erythroid progenitor cells.

In the present study, we investigated the angiogenic potential of peripheral blood-derived erythroid colony-forming cells (ECFCs) and evaluated whether implantation of ECFCs could represent a novel angiogenic cell therapy.

METHODS

Reagents. Recombinant human erythropoietin (rhEPO) was kindly provided by Chugai Pharmaceutical (Tokyo, Japan), whereas recombinant human interleukin-3 and recombinant human stem cell factor (rhSCF) were kindly provided by Kirin-Brewery (Tokyo, Japan). Neutralizing antibodies against VEGF, FGF-2, and transforming growth factor-β (TGF-β) were purchased from R&D Systems (Minneapolis, MN), and a mouse anti-rat CD31 antibody was obtained from BD Biosciences (San Diego, CA). A mouse anti-α-smooth muscle actin (SMA) antibody and the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) color substrates were purchased from Sigma (St. Louis, MO). Fluorescent carbocyanine 1,1'-dioctadecyl-3 to
3,3′,3′-tetramethylindocarbocyanine perchlorate dye was purchased from Molecular Probes (Eugene, OR).

Preparation of ECFCs and PBMNCs. ECFCs were prepared as previously described (31, 32). Briefly, light density mononuclear cells were isolated from heparinized peripheral blood buffy coats (70 ml) from healthy Japanese volunteers by density centrifugation through lymphocyte separation medium (density 1.0770–1.0800 g/ml; ICN Biomedicals, Aurora, OH). Red blood cells were lysed by suspending the mononuclear cell pellet in red blood cell lysis buffer (0.16 mol/l ammonium chloride, 10 mmol/l potassium bicarbonate, and 5 mmol/l EDTA). Platelets were removed by centrifugation in phosphate-buffered saline (PBS) containing 10% human serum albumin (kindly provided by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). At this point, the cells were collected and used as PBMNCs in experiments. Adherent cells were depleted by 1-h incubation in a polystyrene tissue culture flask at 4°C. Nonadherent cells were collected, and negative selection was performed using antibodies against CD3, CD11b, CD15, and CD45RA and immunomagnetic beads in Vario-Macs columns (Miltenyi Biotech, Auburn, CA). The remaining cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco-BRL, Grand Island, NY) containing 15% heat-inactivated fetal calf serum (FCS; Commonwealth Serum, Melbourne, VIC, Australia), 15% pooled human serum, 2 U/ml EPO, 20 ng/ml SCF, and 10 ng/ml IL-3 at 37°C in 5% CO2–95% air in a high-humidity incubator (day 0). On day 3, the cells (referred to as day 3 ECFCs) were centrifuged through lymphocyte separation medium and collected and incubated under the same conditions, except for the absence of SCF. ECFCs were collected on day 3 to day 10 and used in experiments. Homogenous expression of cell surface markers for erythroid differentiation, glycoporphin A and transferrin receptor, were confirmed as previously described by us (21). The morphological purity of the day 6 ECFCs was 95 ± 3%, as determined by cytospin preparations.

Enzyme-linked immunosorbent assays. VEGF production by ECFCs was detected using a Quantikine Immunoassay kit (R&D Systems) according to the manufacturer’s instructions. The intracellular levels of VEGF, FGF-2, tumor necrosis factor-α (TNF-α), and TGF-β in day 6 ECFCs and PBMNCs were detected using specific immunoassays for human VEGF (R&D Systems), FGF-2 (R&D Systems), TNF-α (Japan Immunoresearch, Takasaki, Japan), and TGF-β (Otsuka Pharmaceutical, Tokyo, Japan) according to each manufacturer’s instructions.

Endothelial tubule formation. Tubule formation experiments were conducted in triplicate using an Angiogenesis kit (Kurabo, Osaka, Japan), according to the manufacturer’s instructions as previously reported (6, 17, 40). Briefly, human umbilical vein endothelial cells (HUVECs) and human fibroblasts were admixed and seeded into 24-well plates. For experiments, various numbers of ECFCs were cocultured with HUVECs using cell culture inserts (BD, Bedford, MA) settled in the wells of the plates. Medium containing 2% FCS was supplemented every 3 days. After 10 days of culture, the HUVECs were fixed with 70% ethanol at 4°C and immunostained with an anti-human CD31 antibody using BCP/NTB as the substrate for the secondary antibody. Five fields per well were selected for digital photography under a microscope (Olympus, Tokyo, Japan), and the areas of the tubule-like structures were measured quantitatively using an angiogenesis image analyzer software (Kurabo) (28, 40). Neutralizing antibodies against VEGF, FGF-2, TNF-α, or TGF-β were preincubated with cell-conditioned medium for 60 min, as described previously (11).

An animal model of hindlimb ischemia and transplantation of ECFCs. This study was approved by the Committee on the Ethics of Animal Experiments, Graduate School of Medical Sciences, Kyushu University (Fukuoka, Japan). Hindlimb ischemia was created by resecting the left femoral arteries and veins (7) of immunodeficient nude mice (BALB/c nu/nu; Charles River Japan, Yokohama, Japan) as “an autoamputation model” or nude rats (F344/N nu/nu; CLEA Japan, Tokyo, Japan) as “a limb salvage model” under anesthesia with pentobarbital sodium (50 mg/kg ip). All arterial branches were obliterated by ligation or electrocautery. ECFCs or PBMNCs (1 × 10^7 cells in 50 μl IMDM for mice; 1 × 10^7 cells in 200 μl IMDM for rats) were implanted intramuscularly into the ischemic thigh area (divided into 4 sites) followed by injection of 1,000 IU/kg rhEPO to protect the ECFCs from apoptosis. For the control groups, the same volume of PBS with or without 1,000 IU/kg EPO was injected into the ischemic thigh area.

Limb salvage rate and laser-Doppler analysis. In the autoamputation model, the hindlimbs were photographed at 2 wk after the operation, and the appearances were classified visually into the following three grades: 1) complete salvage (completely normal status
with no signs of ischemia); 2) limb necrosis (necrosis of tissue below the knee); and 3) autoamputation (necrosis of tissue above the knee or loss of the limb), as described previously (15).

In the limb salvage model, a laser-Doppler perfusion imager (Moor Instruments, Devon, UK) was used to measure blood flow in the ischemic and nonischemic limbs.

Immunohistochemistry. Ischemic tissues from the thigh muscles of rats in the limb salvage model were obtained at 4 wk after the operation. Frozen sections (10-μm thickness) were subjected to mouse anti-rat CD31 antibody staining to show the capillary morphology and alkaline phosphatase (ALP) staining by the indoxyl-tetrazolium method to reveal the biochemical activity of the vascular endothelial cells (7, 41). Digital images of 20 fields from two sections were randomly selected from each animal for capillary counts.

Serum concentration of VEGF and FGF-2. Blood samples were collected from rats at postoperative days 0, 1, 3, and 7. After centrifugation, serum samples were subjected to ELISA using Quantikine Immunoassay Systems for human and mouse VEGFs and FGF-2 (R&D Systems) according to the manufacturer’s instructions.

RESULTS

Angiogenic potential of ECFCs. During the in vitro differentiation, VEGF secretion from ECFCs was observed from mice at postoperative days 1, 4, and 7 were minced and homogenized in 1 ml of PBS buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) on ice. After centrifugation, VEGF levels in the supernatants were determined using Quantikine Immunoassay Systems for human VEGF (R&D Systems). Levels of VEGF were expressed according to the muscle weight.

Statistical analysis. The results were expressed as means ± SE. Differences between two groups were analyzed using Student’s t-test. Multiple comparisons among the groups were carried out by one-way ANOVA followed by Bonferroni’s method. The incidence of limb salvage was evaluated by χ²-analysis among the three groups. Data were considered significant at P < 0.05.

Intramuscular content of VEGF. Ischemic thigh muscles obtained from mice at postoperative days 1, 4, and 7 were minced and homogenized in 1 ml of PBS buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) on ice. After centrifugation, VEGF levels in the supernatants were determined using Quantikine Immunoassay Systems for human VEGF (R&D Systems). Levels of VEGF were expressed according to the muscle weight.

Statistical analysis. The results were expressed as means ± SE. Differences between two groups were analyzed using Student’s t-test. Multiple comparisons among the groups were carried out by one-way ANOVA followed by Bonferroni’s method. The incidence of limb salvage was evaluated by χ²-analysis among the three groups. Data were considered significant at P < 0.05.

Fig. 2. Effect of ECFCs on endothelial tubule formation. Human umbilical vein endothelial cells (HUVECs) were cocultured with ECFCs or PBMCs and then immunostained for CD31. A: tubule formation induced by various numbers of ECFCs (4 × 10^2 and 4 × 10^3). VEGF (5 and 10 ng/ml) samples were used as positive controls. B: comparison of the tubule formation induced by ECFCs (4 × 10^3) and PBMCs (4 × 10^3) and the effects of neutralizing antibodies against VEGF, FGF-2, and TGF-β on tubule formation. C: higher-power images of the culture assays in B. Bar, 100 μm. Tf, transferrin; EPO, erythropoietin; SCF, stem cell factor. **P < 0.01 vs. control; †P < 0.05 and ‡P < 0.01 vs. ECFCs.
(corresponding to erythroblasts). As shown in Fig. 1A, ECFCs from days 5 to 7 [corresponding to colony-forming unit erythroid (CFU-E) progenitor cells] possessed the highest VEGF productivity. Therefore, day 6 ECFCs were used in the following experiments. ECFCs also contained various angiogenic factors, such as FGF-2, TNF-α, and TGF-β, in addition to VEGF. The intracellular levels of angiogenic cytokines in day 6 ECFCs were compared with those in PBMNCs. The VEGF content was 3.7-fold higher in ECFCs than in PBMNCs (Fig. 1B; 151.7 vs. 41.0 pg/ml, P < 0.05). The FGF-2 level was also higher (Fig. 1C; 42.7 vs. 30.7 pg/ml), although the statistical significance was marginal. The TGF-β level was significantly lower in ECFCs than in PBMNCs (Fig. 1E; 0.51 vs. 2.91 ng/ml, P < 0.05).

ECFCs surpass PBMNCs for stimulation of tubule formation. In the tubule formation assays, HUVEC were immunostained with an anti-CD31 antibody, and the colored areas were quantified as capillary growth. After coculture for 8 days, the capillary tubule formation induced by ECFCs (4 × 10^5 cells) was 5.2-fold greater than that induced by medium containing 2% FCS (Fig. 2A). A similar magnitude of tubule formation was induced by 10 ng/ml VEGF, EPO, transferrin, and SCF without ECFCs did not significantly affect the tubule formation. Furthermore, the tubule formation induced by ECFCs was 1.5-fold greater than that induced by PBMNCs, and this effect was significantly suppressed by supplementation with antibodies against VEGF (Fig. 2B; 62.3% suppression, P < 0.01) and FGF-2 (Fig. 2B; 32.9% suppression, P < 0.05).

Intramuscular ECFC implantation salvages ischemic limbs from autoamputation. In the autoamputation model using athymic nude mice, the mice develop extensive necrosis or autoamputation of the ischemic hindlimb. We classified the mice according to the degree of ischemia at 2 wk after the operation as shown in Fig. 3. More than one-half of the mice (54%) injected with PBS developed autoamputation, and none of the mice (0%) exhibited complete salvage. As for PBMNC-implanted mice, only 31% exhibited autoamputation, but none of the mice exhibited complete salvage. In contrast, 38% of mice implanted with ECFCs exhibited successful complete salvage, and only 31% developed autoamputation. Statistical analysis revealed a significantly higher rate of complete limb salvage in the ECFC group than in the PBS group or PBMNC group (38% vs. 0%, P < 0.05).

**Enhanced blood perfusion in ischemic limbs implanted with ECFCs.** To investigate blood perfusion in the hindlimbs after implantation of ECFCs, we used athymic nude rats. None of these model rats developed extensive necrosis or autoamputation of the ischemic hindlimb. The rats were implanted with ECFCs (1 × 10^7 cells) followed by EPO (1,000 IU/kg), EPO (1,000 IU/kg) alone or PBS. At 4 days after the operation, limb blood perfusion was severely reduced in all three groups. At 14 days after the operation, significant enhancement of the blood perfusion was observed in the ECFC group compared with the PBS group. (Fig. 4, A and B; 82.88% vs. 54.16%, P < 0.05). Finally, at 28 days after the operation, the ECFC group showed significantly augmented blood perfusion compared with the EPO (82.82% vs. 70.57%, P < 0.05) and PBS (82.82% vs. 65.55%, P < 0.01) groups. No significant differences were observed between the EPO and PBS groups.

**Serum concentrations of angiogenic cytokines.** We measured the serum levels of human VEGF and FGF-2 in the ECFC and PBS groups. Systemic concentrations of human VEGF and FGF-2 were undetectable at days 1, 3, or 7 (data not shown).

Fig. 3. Limb outcomes at 2 wk after the operation in an autoamputation model. Representative macroscopic photographs showing the three different grades classified by the degree of ischemia. Percent distributions of the outcomes were evaluated by χ^2-analysis among mice receiving ECFCs (n = 16), PBMNCs (n = 13), and PBS (n = 13). The incidence of limb salvage was statistically significant.
Intramuscular content of angiogenic cytokines. We measured the levels of human VEGF in the ischemic hindlimbs of the ECFC and PBS groups (Fig. 5). The implanted ECFCs produced human VEGF after the intramuscular implantation. The human VEGF content in homogenized muscle was abundant at day 1 after the implantation (515.4 pg/g muscle). Human VEGF production lasted up to 7 days after the implantation.

Increased capillary density confirms ECFC-induced angiogenesis. To confirm new capillary formation microscopically, we stained frozen sections of ischemic hindlimbs obtained at 28 days after the operation, using three different approaches. Briefly, anti-CD31 staining was used to define the morphology, ALP staining was used to assess the vascular endothelial bioactivity, and anti-α-SMA staining was used to confirm the stability and maturity of vessels (Fig. 6A). According to the anti-CD31 staining, the capillary density was 1.6-fold higher in the ECFC group than in the EPO and PBS groups (Fig. 6B) (389.0/mm² vs. 265.3/mm² and 255.9/mm², respectively, \( P < 0.01 \) for each). No significant differences were observed between the EPO and PBS groups. Significant differences were also found in both the ALP staining and anti-α-SMA staining between the ECFC group and the EPO and PBS groups (ALP: 209.8/mm² vs. 137.8/mm² and 132.4/mm², respectively, \( P < 0.01 \) for each; anti-α-SMA: 179.8/mm² vs. 115.8/mm² and 106.9/mm², respectively, \( P < 0.05 \) for each).

**DISCUSSION**

Various angiogenic factors and cytokines induce angiogenesis and vasculogenesis by collaborative interactions, but the detailed molecular mechanism is not fully understood. VEGF is acknowledged to play a key role in this process and is generally considered to be the most important modulator. It induces the formation of collateral vessels and increases collateral blood flow, leading to improvement in endothelium-dependent vasodilation (5). In addition, it also directly upregu-
contained considerably fewer CD34
supply of angiogenic factors, although the implanted PBMNCs
ischemia and patients with severe ischemia of the lower limbs
EPC implantation has been demonstrated in experimental limb
mobilization from the bone marrow (1). In fact, efficacy of
human umbilical cord blood and are considered to differentiate
EPCs have been discovered in adult peripheral blood as well as
various potent angiogenic cytokines but also EPCs. Circulating
nations of various angiogenic factors may be required for
a smooth muscle cell lining. These results suggest that combi-
that the concerted actions of FGF-2 and VEGF may be neces-
may be insufficient to induce maturation of the capillaries and
(20) reported that overexpressed VEGF in ischemic muscle
adenoviral overexpression of VEGF resulted in the formation
increasing nitric oxide release (33). However, transgenic or
lates endothelial nitric oxide synthase expression, consequently
increasing nitric oxide release (33). However, transgenic or
adenoviral overexpression of VEGF resulted in the formation
of vessels that were hyperpermeable and leaky. Masaki et al.
(20) reported that overexpressed VEGF in ischemic muscle
may be insufficient to induce maturation of the capillaries and
that the concerted actions of FGF-2 and VEGF may be neces-
sary for functional mature neovascularization accompanied by
a smooth muscle cell lining. These results suggest that combina-
tions of various angiogenic factors may be required for
functional mature neovascularization.

Recently, cell therapy for PAD using BMMNCs has pro-
duced feasible angiogenic effects in experimental limb isch-
emia and clinical studies (34, 36). BMMNCs contain not only
various potent angiogenic cytokines but also EPCs. Circulating
EPCs have been discovered in adult peripheral blood as well as
human umbilical cord blood and are considered to differentiate
into endothelial cells and participate in neovascularization after
mobilization from the bone marrow (1). In fact, efficacy of
EPC implantation has been demonstrated in experimental limb
ischemia and patients with severe ischemia of the lower limbs
(12, 15). On the other hand, PBMMNC implantation was also
reported to show effective induction of angiogenesis via the
supply of angiogenic factors, although the implanted PBMMNCs
contained considerably fewer CD34
0.02% vs. 2.4%) (11). This finding raised the possibility that
the supply of angiogenic factors can induce functional mature
angiogenesis without the supply of EPCs.

Previously, it has been shown that BFU-E progenitor cells
express a high level of VEGF mRNA (19, 29) and that
erthroblasts secrete VEGF and placental growth factor pro-
teins during in vitro differentiation (37). In the present study,
we revealed that peripheral blood-derived ECFCs produced
VEGF during in vitro differentiation from BFU-E progenitor
cells to erythroblasts, whereas CFU-E progenitor cells showed
the highest VEGF productivity. ECFCs also showed abundant
production of FGF-2, in addition to VEGF. These results
suggest that erythroid progenitor cells may have an important
role in angiogenesis and also suggest the possibility of novel
therapeutic angiogenesis using erythroid progenitor cells. The
current study represents the first investigation of whether ex
vivo-expanded peripheral blood-derived ECFCs can augment
functional angiogenesis in both in vitro and in vivo models of
critical limb ischemia. The results revealed that 1) ECFCs
stimulated capillary tubule formation in coculture assays with
HUVECs, mainly by supplying VEGF and FGF-2; 2) intra-
muscular implantation of ECFCs significantly increased the
limb salvage rate in an autoamputation model using athymic
nude mice; 3) intramuscular implantation of ECFCs signifi-
cantly increased blood perfusion in a limb salvage model using
athymic nude rats; 4) capillary density increased in rats im-
planted with ECFCs; 5) not only vascular endothelial cells but
vascular smooth muscle cells were increased in ECFC-im-
planted muscle; and 6) the implanted cells survived and pro-
duced VEGF up to 7 days after implantation. These results
demonstrate that ECFC implantation augmented functional
angiogenesis with recruitment of vascular smooth muscle cells
in critical limb ischemia via the cooperative supply of angi-
genic factors, especially VEGF and FGF-2. However, it re-
mained to be evaluated whether ECFCs acted only as a cyto-
kine donor. In addition, elevated cytokines might induce the
mobilization or homing of circulating EPCs (2). The detailed
mechanism requires further investigation.

Given its efficacy, implantation of ECFCs appears to have
several advantages. First, implantation of autologous ECFCs
does not induce toxicity or immunologic rejection compared with
methods involving human recombinant proteins, naked
plasmid DNAs or viruses. Second, ECFCs can easily be ob-
tained from the peripheral blood and expanded ex vivo. The
collection of mononuclear cells or EPCs from the bone marrow
requires general anesthesia, while more than 5–6 liters of
peripheral blood are needed to obtain a sufficient number of
mononuclear cells that are rich in EPCs (39). In the present
study, immature erythroid progenitor cells were partially puri-
fied from peripheral blood by negative selection with antibod-
ies against CD3, CD11b, CD15, and CD45RA and then dif-
fentiated into mature erythroid progenitor cells in the pres-
ence of EPO, SCF, and IL-3. During this ex vivo culture,
immature progenitor cells at day 3 were finally expanded to
almost 15-fold mature progenitor cells at day 6. With the use of
this culture system, large-scale ex vivo amplification for clin-
ic use can be performed to obtain a sufficient number of
erthyroid progenitor cells (8). Third, the ECFCs we used were
not pluripotent, and cell lineage was committed only to ery-
throid. Thus ECFC implantation appears to be very safe since
they finally differentiate into erythrocytes.

In the present study, we coadministered EPO (1,000 U/kg) to
protect the cells from apoptosis since a previous study revealed
that 70% of ECFCs underwent apoptosis within 16 h in
serum-free liquid culture without EPO, compared with only
23% in the presence of EPO (25). EPO is known to be involved
in the cell viability and proliferation of ECFCs (26). Recent
reports have suggested an angiogenic effect of EPO. EPO
stimulated the proliferation and migration of cultured
HUVECs (30), promoted EPC mobilization from the bone
marrow (4, 9), and increased blood perfusion in ischemic
limbs. In contrast with these reports, EPO alone had no
significant effect on capillary tubule formation and did not
significantly augment blood perfusion in the ischemic hindlimb

![Image of a bar graph showing intramuscular content of human VEGF.](http://ajpheart.physiology.org/)

**Fig. 5. Intramuscular content of human VEGF. ECFC-implanted or PBS-
jected muscles ($n = 6$ for each) were obtained from mice at postoperative
days 1, 4, and 7, and the VEGF content in the homogenized muscles was
evaluated by ELISA. The values were expressed as means ± SE; ND, not
detected. $**P < 0.01$ vs. PBS.**
model in the present study. However, it remained possible that EPO promoted EPC mobilization from the bone marrow and augmented ECFC-induced neovascularization. Future studies are needed to address the effects of EPO on in vivo angiogenesis.

In summary, we demonstrated for the first time that intramuscular implantation of peripheral blood-derived ECFCs into ischemic limbs effectively induced functional collateral vessel formation with recruitment of vascular smooth muscle cells via the supply of various angiogenic factors, especially VEGF and FGF-2. ECFCs can easily be obtained from patients, even those with complications of ischemic heart disease, diabetes, or other severe arteriosclerosis and who have a high risk for general anesthesia. This novel angiogenic cell therapy appears to be feasible, although its clinical efficacy should be tested in human trial.

ACKNOWLEDGMENTS
We thank Dr. T. Murohara of the Department of Cardiology, Nagoya University Graduate School of Medicine for excellent comments and advice.

REFERENCES


