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Erythroid cells play essential roles in angiogenesis by bone marrow cell implantation.

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Abstract Bone marrow cell implantation (BMI) has been utilized to treat patients with limb and heart ischemia. BMI provides angiogenic precursors and angiogenic cytokine-producing cells, especially erythroid cells. In this study, we induced *in vitro* angiogenesis cultures and *in vivo* BMI simulation using a murine limb ischemia model to examine the role of erythroid cells and the effect of erythropoietin (EPO). Human erythroid colonies (BFU-e) induced capillary networks around the colonies *in vitro*. Erythroid cells in human bone marrow produced vascular endothelial growth factor and placental growth factor. The angiogenic effects of erythroid cells were further amplified in the presence of EPO. Limb-ischemic mice were treated with BMI $\pm$ EPO, and limb survival, blood flow recovery, and muscle histology were analyzed. Treatment with whole bone marrow cells + EPO significantly improved limb survival and blood flow. The cumulative effects of EPO on BMI induced and increase in capillary number and artery enlargement. Erythroid cells were essential for the *in vivo* effects of BMI, and CD14-positive cells supported the biological effects. In addition to the direct effect of EPO on angiogenesis, EPO showed indirect effect on angiogenesis through amplifying the angiogenic effects by erythroid cells supported by CD14-positive cells.

Keywords: angiogenesis, growth factors, ischemia.

1. Introduction

Organ and tissue ischemia cause severe disorders involving the heart and large blood vessels, central nervous system, limbs, and kidneys, and diseases such as these are targets for angio/vasculogenesis therapy. This involves two major strategies: cytokine therapy, including cytokine and cytokine-gene delivery to induce angiogenesis in residential vessels [1]; and cell-therapy, including blood cell or bone marrow cell implantation to deliver vessel precursors and cytokine-producing cells to ischemic loci [2]. The use of gene therapy involves ethical problems, and in addition, ideal angio/vasculogenesis therapy requires the harmonic combination of angio/vasculogenic cytokines with the time-sequence of vessel regeneration. Peripheral blood includes endothelial cell (EC) precursors (EPC) [3], hematopoietic stem cells, and presumably other angio/vasculogenesis-related cells such as vascular smooth muscle cell (SMC) precursors, and hopefully, common-vascular progenitors developing vessels consisting of EC and SMC [4]. In addition to these cells, bone marrow includes immature hematopoietic cells such as erythroblasts and immature myeloid cells along with a pool of unidentified stem cells that undergo transdifferentiation or gain competence because of cell fusion [5], with cardiomyocytes, hepatocytes, and nerve cells [6].

EPC is present in human blood and bone marrow [3]. Regenerated vessels resulted from vasculogenesis and angiogenesis [7], and this discovery gave rise to the field of "angiotherapoitics" [8]. However, the idea of cell therapy for treating limb and heart ischemia can stand further improvement [9]. Hence, other strategies need to be developed to improve the clinical effect of bone marrow implantation (BMI).

Primary hematopoiesis involving immature erythropoiesis, definitive hematopoiesis that begins in the aorta gonad mesonephros (AGM), and angio/vasculopoiesis, occurs via common progenitors known as hemangioblasts. These cells cooperatively differentiate into nucleated red blood cells and vessels through direct cell-cell contact and the Tie-2/TEK system [10]. In adult bone marrow hematopoiesis, erythropoiesis and angiopoiesis are closely related events. During constitutional vascular remodeling in bone marrow, erythroid progenitors (burst-forming unit-erythroid, BFU-e) arise from hematopoietic stem cells and differentiate into erythroid precursors (colony-forming unit-erythroid, CFU-e). They then lose their migration ability and develop into colonies of immature nucleated red cells in the presence of erythropoietin (EPO). Therefore, immature erythroid

cells present in bone marrow induce the invasion of regenerated vessels into erythroblastic islands. This results in their passive transfer from bone marrow into sinusoidal capillaries, presumably through subendothelial macrophages, after which they undergo simultaneous nuclear discharge. Erythroblasts are a potential source of angiogenic factors in adult human bone marrow [11]. We hypothesized that the simultaneous administration of bone marrow cells and EPO, the main hematopoietic growth factor required for the development of erythroid lineage cells, to ischemic organs might amplify the production of angiogenic factors via implanted erythroblasts extramedullary to the loci, and that this might induce angio/vasculogenesis. To obtain data to support this hypothesis and therapeutic strategy, we studied *in vitro* angiogenesis cultures and *in vivo* BMI using a murine limb ischemia model. This allowed us to examine the role of erythroid cells, the effect of EPO-assisted BMI and its associated cellular and molecular mechanisms.

2. Methods

2.1. Culture conditions

Heparinized blood and bone marrow were obtained from 9 male (ages of 25 to 31 years) and 4 male (ages of 30 to 33) healthy volunteers, respectively, after informed consent was obtained to harvest human hematopoietic colonies and bone marrow cells. Mononuclear cells (MNC) were separated from peripheral blood (PB) and bone marrow (BM) by density centrifugation using a Lymphoprep (1.077, Nycomed Pharma, Oslo, Norway). Erythroid (BFU-e) and granulo/monocytic (CFU-c) colony culturing was performed as reported previously [12]. Each colony was harvested with a sterile pipet tips after 14 days. The colonies were then directly added to an *in vitro* co-culture angiogenesis assay to analyze their effect on angiogenesis.

In vitro angiogenesis assay was performed using an Angiogenesis Kit (Kurabo, Osaka, Japan) according to the manufacturer's instructions, and the formation of capillary-like structures composed of HUVEC (human umbilical vein endothelial cells) co-cultured with human diploid fibroblasts was observed [13]. The medium was exchanged on days 4, 7 and 9, and the cells were washed and directly fixed with 70% ice-cold ethanol for 30 min in the wells after 10 days of culture. Cytokines present in the exchanged medium on day 9 were measured. BFU-e, CFU-c, VEGF-A (final 10 ng/ml, Kurabo), rhEPO (16 or 80 IU/ml), PlGF-1 (10 ng/ml, R&D Systems, Minneapolis, MN), PlGF-2 (10 ng/ml, Relia Tech, Braunschweig, Germany) and angiopoietin-1 (Ang-1, 450 ng/ml, R&D Systems) were tested as angiogenesis stimuli. Mouse anti-human PlGF monoclonal antibody (final concentration: 1 mg/ml, Genzyme, Minneapolis, MN), human recombinant soluble VEGFR1 (sFlt1, 500 ng/ml, R&D Systems), or soluble VEGFR2 (sKDR, 250 ng/ml, R&D Systems) was used to neutralize the effect of PlGF or VEGF in culture. The fixed cells were serially incubated with 1% BSA in buffer, mouse monoclonal antibody against human CD31 (Kurabo), alkaline phosphatase-conjugated goat anti-mouse IgG (Kurabo), BCIP/NBT (violet, Kurabo), and washed and dried. Digital images were acquired using an inverted microscope (Olympus IX70, magnification x 12.5) and video system (Fujix HC-2000, Fujifilm, Tokyo, Japan), whereby the entire field of cultured cells in a well was acquired. The images were analyzed using Angiogenesis Image Analyzer software (Kurabo) to measure the gross area of CD31-positive tubes in culture. In another series of experiments, fixed cells were also serially incubated with 1% BSA, rabbit anti-human VIIIRAg antibody and mouse monoclonal antibody against human CD235a (glycophorin A (GPA), an erythroid marker, Immunotech, Marseille, France), biotinylated goat anti-rabbit IgG (Nichirei, Tokyo, Japan), and FITC-conjugated streptavidin and TRITC-conjugated goat anti-mouse antibody (Dako, Carpinteria, CA). Fluorescence was observed with a microscope (Olympus IX71), confocal laser scan unit (Olympus FV500), and Fluoview software (Olympus). Fluorescent probes were excited with a multi-argon laser (488 nm for FITC) and Green He-Ne laser (543 nm for TRITC). The excited probes were detected with barrier filter sets BA505/525 (FITC) and BA5601F (TRITC), and assigned a color of green or red, respectively. Two-color images were obtained by overlaying images acquired from individual channels for each sample.

The cytokine expression in BMMNC was analyzed in suspension culture. Erythroid (CD235a-positive) and non-erythroid (CD235a-negative) cells were purified from BMMNC using PE-conjugated anti-CD235a (Immunotech) and a MACS magnetic cell-sorting system [14]. The CD235a-positive purity of the samples (n=4) before sorting (MNC), and in the positive (GPA^+) and negative fractions (GPA^-) was 3.9 ± 2.1, 90.3 ± 9.7 and 0.1 ± 0.1 %, respectively. BMMNC, GPA^+ and GPA^- cells were suspended in 2 ml of RPMI1640 supplemented with 10% FBS (1 x 10^6 /ml) in a 6-well plate, and cultured in a CO_2 incubator for 4 days in the presence and absence of rhEPO (final concentration of 10 IU/ml) and rhG-CSF (50 ng/ml, Kirin). The culture supernatant and cells were harvested and analyzed for the production of cytokines and mRNA, respectively. VEGF and PlGF concentrations were measured by ELISA using Quantikine human VEGF and PlGF immunoassay kits (R&D System) according to the manufacturer's instructions.

2.2. RT-PCR and real-time quantitative RT-PCR

RT-PCR was performed to analyze the expression of the mRNA of several angiogenic factors produced by erythroid and granulo/monocytic colonies, as well as by BM erythroid and non-erythroid cells. The primer sets used for VEGF-A, PlGF, HGF (hepatocyte growth

factor), Ang-1, NP-1 (neuropilin-1), PDGF (platelet-derived growth factor), bFGF (basic fibroblast growth factor) and β-actin are shown in Table 1. RT-PCR and real-time quantitative RT-PCR (QRT-PCR) were performed as written previously [15]. The amplification profile for RT-PCR was as follows: 35 cycles at 94°C for 60 seconds, 58°C for 90 seconds, and then 73°C for 120 seconds. QRT-PCR was performed to measure the mRNA expression profiles of VEGF-A and PlGF. The templates were made as control genes to produce standard concentration curves. The cDNA and diluted recombinant plasmid were measured using LightCycler (Roche, Indianapolis, IN) and its software. After initial denaturation for 10 min at 95°C, a 3-step cycle procedure was used (denaturation at 95°C for 10 sec, annealing at 62°C for 10 sec, and then extension and acquisition at 72°C for 13 sec).

2.3. Lower limb ischemia model and bone marrow implantation

Male ICR mice (8 weeks old, 30–35 g) were obtained from Charles River Japan (Yokohama, Japan). All procedures were performed under sterile conditions with the approval of the Institutional Animal Care and Use Committee in compliance with the procedures and methods outlined by the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23; National Institutes of Health, Bethesda, MD). The Animal Study Committee in our institution also approved the experiment projects. The mice were anesthetized by an intraperitoneal administration of ketamine (60 mg/kg) and xylazine (6 mg/kg), and their left femoral arteries were exposed after skin incision. For the limb survival study, the proximal regions of the femoral and saphenous arteries were ligated, they and their branches were removed together, and then the wound was closed. For the analyses of blood flow and histology, the proximal regions of the femoral and saphenous arteries were simply ligated, but not removed, to avoid limb necrosis.

Another mouse was sacrificed by lethal anesthetization, its bilateral femoral bones were removed, and bone marrow was harvested by flushing with culture medium. Mice in each group were injected with 0.2 ml of RPMI 1640 medium (Nipro, Tokyo, Japan) loaded with or without bone marrow cells and EPO intramuscularly into 4 points of an ischemic area (0.05 ml each) using a 23G needle within an hour after ligation. Control mice were injected with medium after ligation (Group A: control). Other mice were injected with 0.2 ml of medium containing recombinant human erythropoietin (rhEPO, 400 IU/kg BW, Kirin, Tokyo, Japan) 0, 24, 48, 72, 96 and 120 hours after ligation (Group B: EPO). Bone marrow cells were washed, and 1 x 10^7 cells in 0.2 ml of medium were implanted (Group C: BMI). Other mice were implanted with 1 x 10^7 cells in 0.2 ml of rhEPO-containing medium within an hour after ligation followed by the injection of 0.2 ml rhEPO-containing medium 24, 48, 72, 96 and 120 hours after ligation (Group D: BMI plus EPO).

To analyze the role of erythroid and other type of cells included in the implanted bone marrow, a discrete subset of cells was positively or negatively sorted from harvested bone marrow using PE-conjugated rat monoclonal antibody against mouse leukocyte antigens and a MACS magnetic cell-sorting system (Miltenyi Biotech, Bergisch Gladbach, Germany) as reported previously [14]. Firstly, population of subsets in bone marrow was analyzed in 5 normal mice using a flow cytometer, and the rates of CD235a (erythroid, Ter119, Becton Dickinson), CD14 (monocyte/macrophage, rmC5-3, Becton Dickinson), and CD34 (stem cells and precursors, RAM34, Becton Dickinson) were 37.4 $\pm$ 10.4 %, 0.30 $\pm$ 0.11, and 0.21 $\pm$ 0.05, respectively. In the groups E and F, 1 x 10^7 of CD235a-negative (non-erythroid) and 0.3 x 10^7 of CD235a-positive (erythroid) bone marrow cells were implanted, respectively. The CD235a % in the implanted cells of groups F and E were 95.5 ± 2.1 % and less than 0.01% (n=13, each), respectively. In the group G, 0.3 x 10^7 of positively sorted bone marrow cells using admixture of anti-CD235a and anti-CD14 were implanted, and the purity of the samples was 90.6 $\pm$ 6.3 % (n=13). In the group H, 0.3 x 10^7 of positively sorted bone marrow cells using admixture of anti-CD235a, anti-CD14 and anti-CD34 were implanted, and the purity of the samples was 96.9 $\pm$ 1.9 % (n=13).

Another series of mice was treated to measure limb blood flow using a moorLDI laser Doppler system (Moor Instruments, Wilmington, Delaware) 6 days after ligation. After measurements were taken, they were sacrificed to obtain the biceps femoris muscles. The muscles were isolated from the surrounding tissue, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at –80°C.

2.4. Tissue preparation and immunohistochemistry

Serial frozen sections (6 μm thick) were applied to silanized slides (Matsunami Glass, Osaka, Japan) and fixed with acetone for 10 min. After blocking with endogenous peroxidase, the slides were serially incubated with rat monoclonal antibody against mouse CD31 (Research Diagnostics, Flanders, NJ), horseradish peroxidase-conjugated goat anti-rat second antibody, and DAB to analyze vascular EC using computer software. To analyze vascular SMC, another series of slides was incubated with mouse monoclonal antibody against human alpha smooth muscle actin (anti-αSMA, which crossreacts with mouse αSMA, Novocastra Laboratories, Newcastle upon Tyne, UK), endogenous mouse immunoglobulin was blocked using a kit (Nichirei, Tokyo, Japan), then visualized using a kit including the second antibody, peroxidase-conjugated polymer and DAB (Histofine Max-POM, Nichirei), and then digitally analyzed. The histology was digitized using a microscope

(magnification x100, Olympus BX60, Olympus, Tokyo, Japan) and video system (Olympus TV System DP50). Digital RGB pictures of the muscle tissues were acquired (1,550 pixel/mm, 2.4 mm^2) and analyzed using MacSCOPE software (Mitani, Fukui, Japan). Briefly, dark brown dots (DAB) were extracted to construct 2 bit pictures, dot clusters less than 5 $pixels^2$ were treated as noise and removed, and the total cluster number, total dot number, and number of muscle fibers were counted. The mean cluster number (cluster number divided by muscle fiber number), mean area (area divided by muscle fiber number in μm^2), and mean vessel size (area divided by cluster number in μm^2) were calculated as representatives of vessel number, vessel area, and mean vessel size, respectively.

2.5. Statistical analysis

The means and standard deviations (SD) were calculated and used to express the results for the experiments. The values were compared among groups by one-way ANOVA followed by the Fisher multiple comparison test, and the results for the *in vitro* experiments were compared by the Student's paired *t*-test. Survival and recovery curves for the lower limb after treatment were calculated using the Kaplan-Meier method and compared by the generalized Wilcoxon test.

3. Results

3.1. Angiogenesis culture in vitro and factor production by hematopoietic cells

The expression profile of angiogenic factor mRNA in human PB-derived hematopoietic colonies and human bone marrow cells were analyzed by RT-PCR for 3 individuals for each factor. PBMNC before culture, BMMNC, BM erythroid cells, and BM non-erythroid cells expressed VEGF, HGF, Ang-1, NP-1, PDGF and bFGF (Fig. 1). PlGF was expressed in PB-derived erythroid colonies and BM erythroid cells. Both erythroid and granulo/monocytic colonies expressed VEGF, NP-1, PDGF and bFGF. HGF and Ang-1 mRNA were observed in BM erythroid cells but BFU-e was not. Although the purity of sorted bone marrow erythroid cells was high, the mRNA of HGF and Ang-1 might have come from contaminated non-erythroid cells, or erythroid colonies in culture may have lost the ability to produce HGF and Ang-1 under artificial conditions. We focused on the production of VEGF and PlGF as a ubiquitous angiogenic factor and an erythroid-restricted factor, respectively.

The effects of BFU-e and CFU-c on angiogenesis are shown in Figs. 2 and 3. BFU-e enhanced angiogenesis in a dose-dependent manner ($p = 0.038$ between groups "BFU-e x 3" and " x 10" in Fig. 3, paired *t*-test), and the addition of EPO seemed to further enhance this effect ($p > 0.05$). On the other hand, CFU-c produced no effect. Drops of semisolid complete medium for colony formation did not affect angiogenesis (data not shown). VEGF and Ang-1 exhibited strong angiogenic effects, while the effects of PlGF and EPO were weak. The addition or absorption of PlGF did not alter the effect of BFU-e, while the neutralization of VEGF using sVEGFRs blocked angiogenesis in both the presence and absence of BFU-e.

The supernatant harvested from the control culture contained VEGF and PlGF, which was presumably produced by fibroblasts. The presence of BFU-e in culture indicated the cumulative production of VEGF and PlGF, and production was further stimulated in the presence of EPO. CFU-c did not affect the production of VEGF and PlGF (data not shown). Taken together, the effect of BFU-e on the capillary tube formation by EC did not occur due to PlGF, but rather due to VEGF and possibly other factors produced by the erythroid colonies.

As shown in Fig. 4, human bone marrow cells produced VEGF and PlGF in the suspension culture. Erythroid cells produced both cytokines, and production was further stimulated by EPO. Non-erythroid cells produced trace levels of VEGF and PlGF.

3.2. *In vivo* effect of BMI and EPO

A harsh limb ischemia model was utilized for the limb survival study. ICR mice are sensitive to ischemia, and their femoral arteries were thoroughly removed. Fifty-two mice (13 each in Group A, B, C, and D) were observed to analyze the recovery and survival of ischemic limbs. Cyanosis was observed for all treated limbs within 24 hours, where some limbs recovered and others developed partial or total necrosis except one mouse in Group B in which cyanosis continued for 28 days (Fig. 5). Limb necrosis was observed within 7 days after treatment, while recovery from cyanosis was observed subsequently. Significant differences were observed for the recovery and survival curves among the 4 groups.

In another series of experiments, limb blood flow was estimated 6 days after ligation by the flux ratio (flux in ischemic limb/flux in the other lower limb) measured using a laser Doppler system for Groups A ($n = 7$), B (5), C (7) and D (5) as shown in Fig. 6. A significant difference was observed between Groups A and D. Immunohistochemistry was analyzed for 7 (Group A), 6 (B), 5 (C) and 6 mice (D). Most of the regenerated vessels were not accompanied by SMC, and the vessel number increased in Group D (cluster number in Fig. 7), and the gross area of EC and SMC increased for Group D (area in Fig. 7). The mean EC area did not differ between the groups, so the majority of vessels, SMC-absent capillaries, increased in number but were not enlarged by BMI or EPO-assisted BMI. On the other hand, the mean SMC area was dramatically enlarged by EPO-assisted BMI, so that the minority of regenerated vessels accompanied by SMC was enlarged and blood flow recovered as a result. Implanted erythroid cells were also stained using Ter119 antibody, however, they were not observed in

the specimens. The cells might not have survived extramedullary as long as 6 days after possibly triggering angiogenesis in vivo. In summary, the main effects of EPO-assisted BMI on angio/vasculogenesis *in vivo* increased the capillary number and enlarged the SMC-associated arteries, probably as a result of the effects of VEGF on EC and PlGF on SMC, respectively.

3.3. Roles of bone marrow subsets

For estimating the roles of erythroid and other type of cells *in vivo*, another series of fifty-two mice (13 each in Group E, F, G, and H) were observed to analyze the recovery and survival of ischemic limbs (Fig. 8). Limbs did not survive in mice transplanted with erythroid-depleted cells, hence bone marrow erythroid cells were essential for the effect of BMI (Group E). Limbs did not survive unexpectedly in mice transplanted with purified erythroid cells, therefore accessory cells were estimated to assist the effect of erythroid cells (Group F). The BMI effect recovered in Group G, so the major accessory population was CD14-positive. The additional effect of CD34-positive cells was not powerful (Group H).

Discussion

Several resources of cells such as peripheral blood mononuclear cells (PBMNC), bone marrow cells (BMMNC) and marrow-derived stromal cells (MSC) have been utilized for angiogenesis to treat ischemic arterial diseases. Among them, like bone marrow erythroid cells, MSCs also produced several angiogenic cytokines including especially VEGF and PlGF, and developed mature vessels in a murine limb ischemia model [16]. For mimicking the treatment of acute arterial obstruction or chronic perfusion insufficiency, cell delivery is undergone in a couple of days or after 3 or more weeks of surgical ligation in animal ischemia models, respectively. The BMI undergone within an hour after femoral ligation rescued ischemic limbs in our study, while MSC injection immediately after ligation failed to improve flow recovery [16]. The reason is not known what the difference between BMI and MSC implantation is. The survival of extramedullary implanted cells in a ischemic loci may differ. The crude admixture of bone marrow cells may tend to survive through mutual protection of bone marrow subpopulations, while the purified and naked MSC may not survive in such a harsh storm of cytokines occurred just after the sudden obstruction of large arteries. The simultaneous administration of EPO with BMI virtually protected erythroblasts from apoptosis and necrosis in the ischemic loci, and prolonged the production of angiogenic factors by the cells.

In this study, bone marrow erythroid cells and erythroid colonies strongly produced VEGF and PlGF, and the addition of EPO further stimulated this production. The addition of erythroid colonies or VEGF in culture promoted the enhanced formation of EC tubes *in vitro*. The effect of erythroid colonies was presumably the result of VEGF production, but not PlGF, since the addition or neutralization of PlGF did not affect the growth of EC tubes. EC tube formation by erythroid colonies was also further enhanced by the addition of EPO through an indirect effect on the erythroid cells, because EPO alone showed little direct effect. The cumulative effects of EPO on BMI therapy were the improvement of ischemic limb survival and recovery of blood flow through vascular regeneration. EPO-assisted BMI strongly enhanced capillary formation and vascular SMC development, presumably through VEGF and PlGF, respectively. Size and not the number of SMC-associated arteries increased due to combination therapy, and this effect resulted in the improvement of blood flow in ischemic loci. Implantation of erythroid-depleted bone marrow cells did not rescue limb ischemia, therefore the existence of erythroid cells in the implant played essential roles for recovery from ischemia. In a Japanese clinical trial study of BMI in patients with peripheral artery disease, mature neutrophils (%) and erythroblasts (%) in the implanted bone marrow showed negative and positive correlations, respectively, with the improvement of ABI and tissue oxygen pressure [17].

However, the implantation of purified erythroid cells also did not rescue limbs. The most expected reason for this was that an accessory cell population exists in bone marrow that is effective in angiogenesis such as CD14-positive cells. CD14 is one of the receptors for LPS and is expressed in most blood monocytes. In bone marrow hematopoiesis, mature erythroblasts in erythroblastic islands are passively transferred to peripheral circulation through subendothelial macrophages, therefore CD14-positive cells in bone marrow may assist angiogenesis by erythroid cells. In fact, the addition of CD14-positive cells (surprisingly only 0.3% in bone marrow) recovered the effects of purified erythroid cells *in vivo*. Other candidate cells assisting angiogenesis include bone marrow CD34-positive cells that contain hematopoietic precursors, EPCs, and stem cells. However, the effect of further addition of CD34-positive cells was not so strong. Therefore, the effects of BMI may arise mainly from angiogenesis.

Bone marrow cells expressed the mRNA of several angiogenic cytokines, including VEGF, PlGF, HGF, PDGF, bFGF and Ang-1, and among them, PlGF was specifically expressed in erythroid bone marrow cells. For the *in vitro* angiogenesis culture of EC, the concentration gradient of VEGF around erythroid cells along with other cytokines may explain the cooperative growth and gathering of EC tubes around erythroid colonies. Analogous to this explanation, the concentration gradients of VEGF, PlGF and other cytokines produced and expressed by EPO-stimulated bone marrow cells may explain the concerted angiogenesis observed *in vivo* for EPO-assisted BMI.

In the human placenta, VEGF and PlGF are produced by different type of cells [18]. On the other hand, erythroblasts produce both cytokines, therefore, are expected to produce VEGF/PlGF heterodimer. The heterodimeric cytokine gives rise to strong signal in cells through heterodimerization of VEGFR1 and 2 [19].

EPO is constitutively produced to maintain erythropoiesis, and is reactively produced in response to hypoxia [20]. The EPO/EPO-R system also has other pleiotropic functions such as inducing angiogenic [21] and anti-apoptotic effects [22], and EPC mobilization [23]. In the cardiovascular system, EC, SMC and cardiomyocytes express EPO-R, and EPO induces growrh and anti-apoptotic effect in the cells through JAK2/STAT5 and PI3-K pathways [20]. In addition to the direct effect of EPO on angiogenesis as well as vasculogenesis through migration and proliferation of mature EC, and mobilization and survival of EPCs, we hypothesized that the simultaneous administration of EPO with BMI works as an angiogenesis co-stimulator mainly through its effect on erythroid cells. A direct effect of EPO was also observed in this study. From the histological findings, the administration of EPO alone increased the capillary number and area equally to that induced by BMI; however, it had no effect on SMC in vivo. The putative effects of EPO in hematopoiesis are as follows: 1. Helping the commitment of hematopoietic stem cells to the erythroid lineage; 2. Inducing the proliferation and differentiation of erythroid cells, and possibly; 3. Further activating erythroid cells to produce several angiogenic factors, resulting in angiogenesis.

Basic and clinical studies on G-CSF administration and G-CSF-assisted cell therapy have been reported [24], with encouraging results in rodent models, but in contrast, raised concern in the clinical trials, where it improved cardiac function but progressed post-PTCA restenosis [25]. The safety and adverse effects of EPO administration are well-known from clinical use. EPO is water-soluble, and is quickly cleared from injected tissues, therefore modification of the drug delivery system [26] or a new molecular derivative of EPO is necessary to maintain tissue concentration. Secondary polycythemia is another problem associated with EPO administration. As a primary study, clinical trials involving EPO-assisted BMI for patients with limb ischemia complicated by Atherosclerotic obliterance and Burger's disease as well as acute and chronic myocardial infarction has been approved by the Ethical Committee of Niigata University Medical and Dental Hospital. The result will be published in the future.

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Table 1 Primers for RT-PCR

VEGF-A	5'-ATGAACTTTCTGCTGTCTTGGGT
	3'-GCTCTATCTTTCTTTGGTCTGCAT
PlGF	5'-GTCATGAGGCTGTTCCCTTG
	3'-CAGAACGGATCTTTAGGAGCTG
HGF	5'-AATAAAGGACTTCCATTCACTTGC
	3'-AGGATTTCGACAGTAGTTTTCCTG
Ang-1	5'-GTTGGACACCTTAAAGGAAGAGAA
	3'-ATTGACATCCATATTGCAAAACAC
NP-1	5'-GTTACTGTGGACAGAAAACACCAG
	3'-CCCAAGTCTACCTGTATCCACTCT
PDGF-A	5'-GTAGGGAGTGAGGATTCTTTGGAC
	3'-TGGACTTCTTTTAATTTTGGCTTC
PDGF-B	5'-CGAGTTGGACCTGAACATGAC
	3'-CCTTCTTAAAGATTGGCTTCTTCC
bFGF	5'-AAGCGGCTGTACTGCAAAAAC
	3'-AAGTTTATACTGCCCAGTTCGTTT
β-actin	5'-ATCATGTTTGAGACCTTCAA
	3'-CATCTCTTGCTCGAAGTCCA

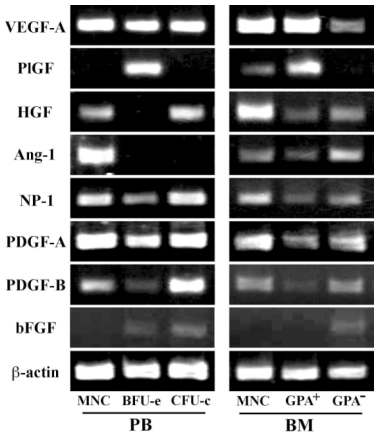

Fig. 1 The expression of mRNA of angiogenic factors in human bone marrow mononuclear cells (MNC), purified bone marrow erythroid (GPA^+) and non-erythroid (GPA^-) cells, and peripheral blood-derived erythroid (BFU-e) and granulo/monocytic (CFU-c) colonies. VEGF, NP-1, PDGF and bFGF were ubiquitous, while PlGF was erythroid-restricted.

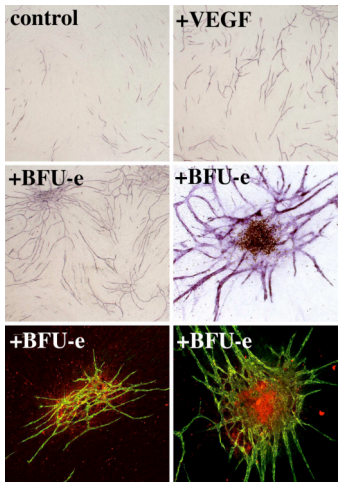

Fig. 2 Capillary network formation in human angiogenesis cultures is shown. Upper 4 pictures: HUVEC stained by anti-CD31 and BCIP/NBT, and lower 2 pictures: HUVEC and erythroid cells stained by anti-VIIIRAg-FITC (green) and anti-CD235a-TRITC (red), respectively, in the absence (control) or presence of VEGF or 10 BFU-e. Magnification: upper 2 and middle left, x20; middle right, x100 (inverted microscope); lower 2, x100 (confocal microscope). Note that hematopoietic colonies were washed to the fringe of the wells by medium convection currents, and capillary networks (purple) collected and grew around the erythroid colonies (dark red hemoglobin).

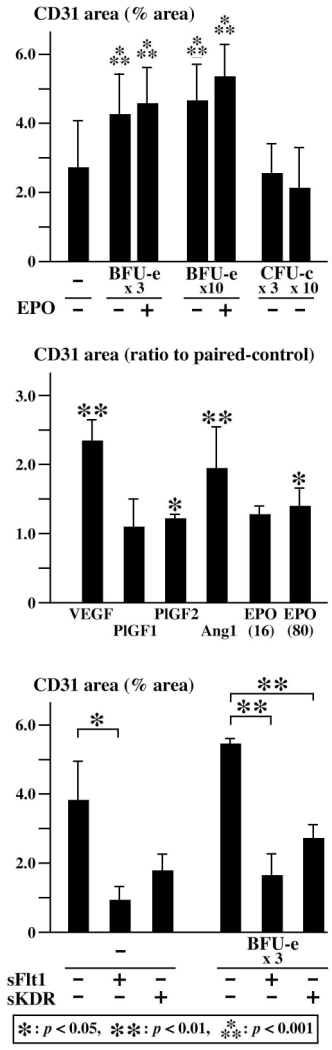

Fig. 3 The CD31-positive capillary-like structures of EC tubes in Fig. 2 were digitized for quantitative analysis. The upper graph shows the effects of human blood-derived erythroid (BFU-e) or myeloid colonies (CFU-c) added in the culture in the presence or absence of rhEPO (16 IU/ml) (n=6). The capillary network formation was supported by the addition of BFU-e, but not by CFU-c. The middle graph shows the effects of several cytokines (VEGF, PlGF1 and PlGF2, 10 ng/ml; Ang1, 450 ng/ml; EPO, 16 or 80 IU/ml) (n=5). VEGF and Ang-1 strongly induced angiogenesis, while the effects of PlGF and EPO were weak. The lower graph shows the cytokine-neutralization analysis (n=3). Additions of sFlt1 (soluble VEGFR1, 500 ng/ml) or sKDR (soluble VEGFR2, 250 ng/ml) significantly blocked angiogenesis. Additions of PlGF1, PlGF2 or anti-PlGF antibody (1 μg/ml) did not affect angiogenesis in the presence of BFU-e (data not shown). *The upper and lower graphs represent the CD31-positive area divided by the whole area. The middle graph represents the CD31-positive area of samples divided by that of paired-control cultures.

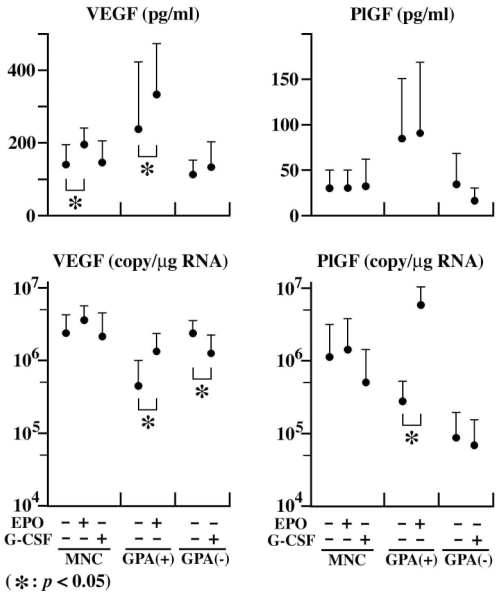

Fig. 4 The cytokine production (upper graphs) and mRNA expression (lower) of VEGF and PlGF by human bone marrow cells are shown (n=4). VEGF and PlGF were strongly produced by GPA-positive erythroid cells. VEGF production was further enhanced by the addition of EPO (16 IU/ml).

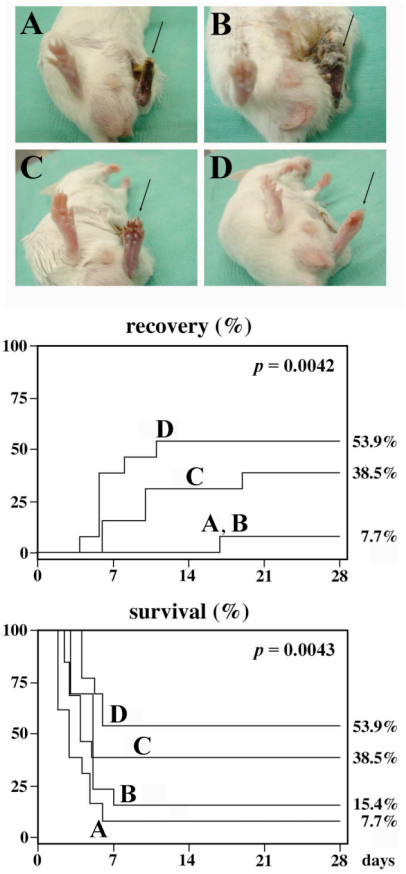

Fig. 5 Ischemic limbs observed on day 7 (photos), recovery from cyanosis, and survival of limbs after ligation (n = 13 in each group). Groups A, B, C, and D: injected with control medium, erythropoietin (2,400 IU/kg), bone marrow cells (1 x 10^7), and both, respectively. Simultaneous administration of erythropoietin supported the effects of BMI. Difference was significant among the 4 groups by Wilcoxon test.

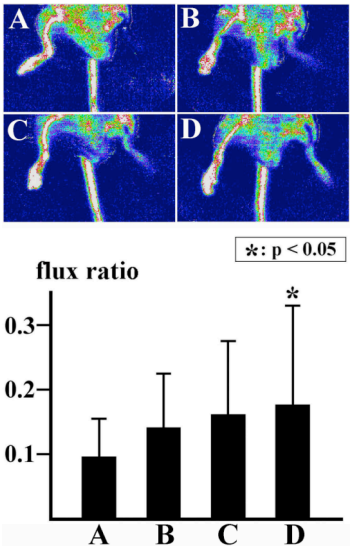

Fig. 6 Images acquired by a laser Doppler system, and the flux ratio on day 7 after ligation. Groups A, B, C, and D: injected with control medium, erythropoietin (2,400 IU/kg), bone marrow cells (1 x 10^7), and both, respectively. The flux ratio for Group D was significantly higher than that for Group A.

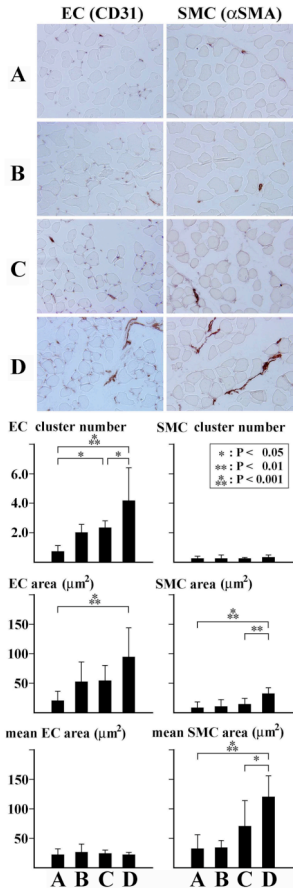

Fig. 7 Histology of biceps muscles on day 7 after ligation. Endothelial cells (EC) and smooth muscle cells (SMC) were stained with anti-CD31 and anti-αSMA, respectively, visualized with DAB (magnification, x 200), and digitized for quantitative analysis (ratio to muscle fiber number). Groups A, B, C, and D: injected with control medium, erythropoietin (2,400 IU/kg), bone marrow cells (1 x 10^7), and both, respectively. Most vessels were not accompanied by SMA, and the vessel number increased for Group D (cluster number). The total area of EC and SMC significantly increased for Group D. The mean SMC area, but not EC, increased in size for Group D. The total area of the analyzed field was 2.4 mm^2 each.

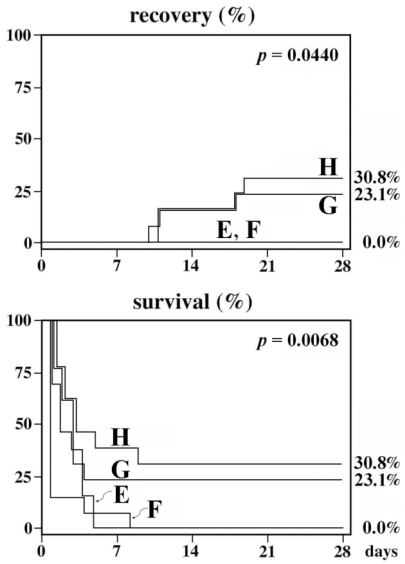

Fig. 8 Recovery from cyanosis, and survival of limbs after implantation of bone marrow subsets (n = 13 in each group). Groups E and F: injected with erythroblast-depleted bone marrow cells (1 x 10^7 GPA-negative cells) or purified erythroblasts (0.3 x 10^7 GPA-positive cells). Group G: injected with 0.3 x 10^7 of purified erythroblasts and macrophages (CD14-positive). Group H: injected with 0.3 x 10^7 of purified erythroblasts, macrophages, and stem cells (CD34-positive). As written in the text, proportions of GPA-, CD14-, and CD34-positive cells in the implanted bone marrow were roughly 1/3, less than 1%, and less than 1%, respectively. Then, for equalizing the implanted cell number of the subpopulations, cells were adjusted as written above in each group. Erythroblasts were essential for the effects of BMI, and the cells worked in the presence of macrophage. Further effects of the addition of stem cells were not so strong. Difference was significant among the 4 groups by Wilcoxon test.