J Pharmacol Sci **112**, 167 – 175 (2010)

Journal of Pharmacological Sciences

Full Paper

Effect of Erythropoietin on Angiogenesis With the Increased Adhesion of Platelets to the Microvessels in the Hind-Limb Ischemia Model in Mice

Shintaro Kato[1,2], Hideki Amano[3], Yoshiya Ito[4], Koji Eshima[5], Naoyoshi Aoyama[1], Hideaki Tamaki[6], Hiroyuki Sakagami[6], Yukitoshi Satoh[3], Toru Izumi[2], and Masataka Majima[1,*]

Departments of [1]Pharmacology, [2]Cardio-angiology, [3]Thoracic Surgery, [4]Surgery, [5]Immunology, and [6]Anatomy, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara 228-8555, Japan

Received September 19, 2009; Accepted November 24, 2009

Abstract. Erythropoietin (EPO) has been shown to enhance angiogenesis, but its precise mechanisms of enhancement during ischemia are not fully elucidated. We examined the effect of EPO on blood flow recovery from acute hind-limb ischemia induced by ligation of the femoral artery in male C57Bl/6 mice. The density of microvessels with platelet adhesion in ischemic tissues was assessed by intravital microscopy. Treatment with EPO (100 and 1000 IU/kg, i.p.) restored blood flow in a dose-dependent manner and increased plasma levels of soluble-P-selectin, vascular endothelial growth factor (VEGF), and stromal cell–derived factor (SDF-1). Flow cytometric analysis revealed increased P-selectin expression on platelets in EPO-treated mice compared to PBS-treated mice. Intravital microscopic studies showed that EPO increased density of microvessels with platelet adhesion selectively in the ischemic tissues. Neutralizing antibody against P-selectin reduced the density of microvessels with platelet adhesion enhanced with EPO and impaired blood flow recovery with reductions in VEGF and SDF-1 levels. These results suggest that EPO administration enhances recovery from hind-limb ischemia, and platelet adhesion to the microvessels is a key event to enhance the angiogenesis in the ischemic tissues.

Keywords: angiogenesis, erythropoietin, intravital microscopy, platelet, P-selectin

Introduction

Angiogenesis is a complex process that involves the activation of vascular cells through a balance of pro- and anti-angiogenic factors. Erythropoietin (EPO) is a hematopoietic cytokine that is known to promote proliferation and differentiation of erythroid progenitors. Besides hematopoietic activity, EPO enhances the mobilization of endothelial progenitor cells (EPCs) (1) and exerts a protective effect on endothelial cells in several vascular injury models partly through suppression of apoptosis (2). These suggest that EPO may enhance the recovery from ischemia.

Platelets play important roles in hematostasis and thrombus formation, but it has been shown that platelets also participate in inflammation and tissue repair through paracrine pathways or direct cell–cell interactions (3). The immediate appearance of platelets at the site of injured vascular wall indicates that platelets would be an important trigger for angiogenesis and tissue regeneration (4). The potential role of platelets in angiogenesis has been reported by in vitro studies demonstrating that platelets promote survival and proliferation of endothelial cells and angiogenesis (5). Systemic platelet transfusion and local injection of platelets into an ischemic limb promote recovery from reduction in blood flow in a model of hind-limb ischemia (6, 7). On the other hand, depletion of platelets suppresses blood flow recovery (6). Activated platelets are a major source of angiogenic growth factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), stromal cell–derived factor (SDF)-1, and fibroblast growth factor (FGF)-2 (8).

Since EPO is reported to activate platelets (9 – 11),

*Corresponding author. mmajima@med.kitasato-u.ac.jp
Published online in J-STAGE on February 4, 2010 (in advance)
doi: 10.1254/jphs.09262FP

EPO may enhance angiogenesis in a platelet-dependent manner. We had previously reported that intravital microscopy is a very useful method to reveal the interaction of endothelial cells with circulating cells including platelets (12). In the present study, we tested whether the exogenous EPO enhances angiogenesis in acute hind-limb ischemia via enhancement of the interaction of platelets and endothelial cells using in vivo microscopic methods. Furthermore, we identified the relevant adhesion molecule to maintain the interaction between the endothelial cells and platelets.

Materials and Methods

Animals and surgery

The model of hind-limb ischemia was previously described (6). Male C57Bl/6 mice (8 weeks of age) were obtained from Clea Japan (Tokyo). Under anesthesia with pentobarbital sodium (50 mg/kg, i.p.), a midline incision was made in the abdominal skin, permitting dissection to expose the external iliac artery in the upper part of the left limb. The artery was then ligated both proximally and distally using 6-0 silk suture and the intervening 6-mm section excised. Then, the incision was closed. All mice were kept at constant temperature (25 ± 1°C) and humidity (60 ± 5%) with free access to normal chow and water throughout the experimental periods. The study was performed in accordance with the guideline for animal experiments of Kitasato University School of Medicine.

Experimental protocols

Human recombinant EPO (100 or 1000 IU/kg) (Chugai, Tokyo) (13, 14) or PBS was injected intraperitoneally three times per week, that is, at 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 days after surgery. There were six animals in each group. The doses used here were reported to induce hematopoiesis in mice (13, 14). There was a previous report in which systemic blood pressure was not increased after 1000 IU/kg EPO (s.c.) administration (15). In a separate set of experimental animals, EPO (1000 IU/kg) or PBS was administered given before surgery, and blood was collected from the tail for measurements of soluble-P-selectin (s-P-selectin), VEGF, and SDF-1 at indicated time intervals (n = 6 / time point).

The monoclonal antibody to P-selectin (RB40.34, rat IgG1, 30 μg per mouse; BD PharMingen, San Jose, CA, USA) or vehicle (rat IgG, BD PharMingen) was injected (100 μl) every day into the mice treated with ten administrations of EPO (1000 IU/kg) according to the same protocol as described above.

Laser Doppler imaging (LDI)

Blood flow to the right and left hind limbs was assessed by scanning the lower abdomen and limbs of the mice with a Lisca (PIM II) scanning laser Doppler (Perimed, Järfälla, Sweden). The ratio of blood flow in the ischemic (left) to the control (right) limb was calculated by dividing the integrated blood flow in an area of the image that included the left foot pad by the integrated blood flow for an area of the same size that included the right foot pad. During the experiment, the raw blood flow values of the non-ligated hind-limb (the control limb) was not markedly changed. Blood flow measurements were assessed by scanning both pre- and postoperatively and on days 3, 7, 14, 21, and 28.

In vivo microscopy

Seven days after surgery, animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and were prepared for in vivo fluorescence microscopy according to modifications of methods previously described (16). Hind-limb microcirculation was observed using a fluorescence microscope (ECLIPSE E600, upright type; Nikon, Tokyo) with a 100-W mercury lamp for epi-illumination. The microscopic images were obtained with a long working distance objective lens (M plan 40/0.40 SLWD, Nikon) and an X10 eyepiece lens. Images of the microcirculation were transmitted through a CCD camera (C7190; Hamamatsu Photonics, Hamamatsu) to a TV monitor screen (PVM-144Q; Sony, Tokyo) and were recorded for subsequent off-line analysis on videotape with an S-VHS recorder (BR-S600; Victor, Tokyo). Platelets were labeled with 0.3 μmol/kg of rhodamine-6G (Sigma, St. Louis, MO, USA) (i.v.) just before the start of the observation. The microcirculation through the terminal arterioles, capillaries, and venules in the quadriceps muscles or through the tissues adjacent to the femoral artery and vein was observed in mice treated with EPO (1000 IU/kg, n = 4) or PBS (n = 4). In a separate set of experimental animals, an anti–P-selectin antibody (n = 4) or vehicle (n = 4) was daily administered to the mice treated with EPO (1000 IU/kg) according to the same protocol as described above.

To examine the interaction of platelets with endothelium, platelet adhesion to vessels at the same optical place on the surface of the hind-limb muscle tissues was analyzed in ten peri-femoral or ten muscular regions in each animal. The total length of microvessels on which with rhodamine 6G-labeled platelets were deposited per observation area was measured. The results were averaged, and the data were expressed as density of microvessels with platelet adhesion (μm/10,000 μm^2).

Blood hematocrit (Ht) and platelet numbers

Blood was drawn via the tail vein before and 1, 2, 3, 7, 10, 14, 21, and 28 days after surgery. The collected amounts of blood were 20 μl at each time point. Ht and platelets were measured by Celltac α (MEK-6450; Nihon Kohden, Tokyo).

Flow cytometric analysis

In some experiments, EPO (1000 IU/kg, n = 4) or PBS (n = 4) was intraperitoneally administered on 0, 2, 5, and 8 days after surgery. A 100-μl sample of blood was drawn via the tail vein on 0, 1, 4, 7, and 9 days after surgery. The white blood cell fraction including platelets was obtained by ficoll separations Flow cytometric analysis was performed as described previously (14). Cells were labeled with FITC-labeled anti-CD41 Ab (Bioscience, San Diego, CA, USA) and PE-labeled anti–P-selectin Ab or isotype control Ab, in the presence of anti-FcR 2.4G2 (Becton Dickinson, San Jose, CA, USA). After washing, cells were analyzed with FACS Calibur (Becton Dickinson) and small cells (low for FSC) were gated for platelet analysis. In a separate experiment, we used anti-EPO receptor (EPOR) antibody [EpoR (M-20): sc-697; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PE (sc3739)–conjugated goat anti-rabbit IgG to see EPOR-positive platelets.

Concentrations of VEGF, SDF-1, and s-P-selectin in plasma

To determine plasma levels of VEGF, SDF-1, and s-P-selectin, the blood (100 μl each in volume) was collected using microhematocrit capillary tubes (Fisher Scientific, Pittsburg, PA, USA). The blood was transferred to a tube and centrifuged for 20 min at 2000 × *g* within 30 min of collection. The plasma was collected and stored at −20°C until assay. Plasma levels of VEGF and SDF-1 at 3 days after surgery and of s-P-selectin at 0, 1, 3, 5, and 7 days after surgery were assessed with ELISA specific for murine VEGF, SDF-1, and s-P-selectin (R&D Systems, Minneapolis, MN, USA)

Immunofluorescence

For immunofluorescence, the tissue samples from ischemic hind limb were fixed with 10% neutral buffered paraformaldehyde at 4°C for 1h. Following cryoprotection with 30% sucrose / 0.1 M phosphate buffer (pH 7.2), cryostat sections about 10 – 20 μm in thickness were cut, and non-specific staining was blocked by the incubation with 1% bovine serum albumin (BSA) / PBS for 1 h. The sections were incubated with a mixture of primary antibodies in 1% BSA / PBS at room temperature for 1 h or overnight. Primary antibody for EPOR [EpoR (M-20): sc-697] was mounted on the sections. After being washed three times in PBS, the sections were incubated with a mixture of secondary antibodies for 1 h at room temperature. The secondary antibody was Alexa Fluor 488 conjugated with anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR, USA). They were then observed using a confocal scanning laser microscope (LSM710; Carl Zeiss MicroImaging GmbH., Oberkochen, Germany). Serial optical sections (collected at 1-μm intervals) in the z-axis were collected and overlaid for the final images shown using ZEN-2008 software (Carl Zeiss MicroImaging GmbH.) installed on LSM710.

Morphological quantification of blood vessel formation

Capillary density in the ischemic muscles tissues was assessed as a parameter of angiogenesis in a blind manner according to the methods described previously (6). The numbers of CD31-positive cells were counted in 20 randomly selected microscopic fields from 5 different transverse sections in each animal. The results were averaged, and capillary density was expressed in terms of the number of CD31-positive cells per mm^2.

Statistics

Data are expressed as means ± S.D. Comparisons among multiple groups were performed by analysis of variance (ANOVA). Comparisons between the two groups were made by Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

EPO enhances recovery from hind-limb ischemia and increases capillary density

To confirm that EPO is a critical player in angiogenesis, the impact of EPO administration on the recovery from hind-limb ischemia was examined. Hind-limb ischemia resulted in decreased blood flow ratio (ischemic/control limb), as assessed by laser Doppler scanning from 0.97 ± 0.03 to 0.11 ± 0.01 immediately after surgery (Fig. 1A). Ten administrations with EPO (1000 IU/kg) on days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 days after surgery increased recovery of flow ratio by day 28 from 0.63 ± 0.07 with PBS to 0.68 ± 0.05 with EPO (100 IU/kg) and 0.91 ± 0.08 with EPO (1000 IU/kg) ($P < 0.005$). Systemic blood pressure was not increased in all the points by EPO treatment.

Sections of quadriceps in the ischemic limb were stained with an anti-CD31 antibody to identify endothelial cells (Fig. 1: B and C). The average number of CD31-positive cells per muscle fiber in EPO (1000 IU/kg)–treated mice (2.61 ± 0.191) was significantly higher than that in PBS-treated mice (2.28 ± 0.244) (Fig. 1D). There was no significant difference in capillary density

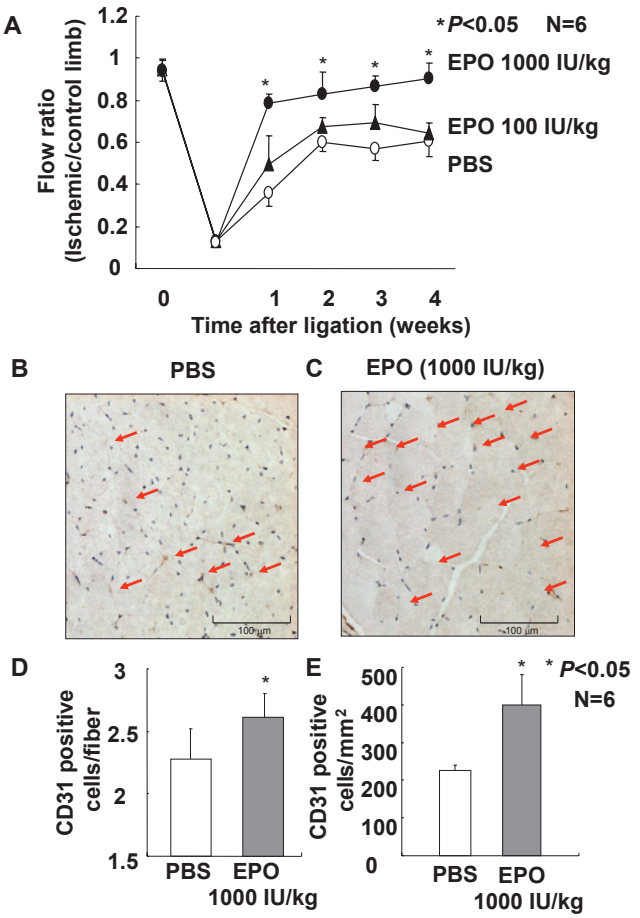

Fig. 1. Effects of EPO treatment on blood flow recovery from hind limb ischemia and capillary density. Ischemia was induced in the left limb of C57Bl/6 mice. On days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 after surgery, the mice received i.p. injection of EPO (100 IU/kg), EPO (1000 IU/kg), or PBS. A) Blood flow ratio was assessed by laser Doppler scanning before and at intervals after surgery. Data are expressed as means ± S.D. from 6 mice per group. B and C) Photographs of ischemic muscle sections stained with an anti-CD31 antibody from mice treated with PBS (B) and EPO (1000 IU/kg) (C) 7 days after the hindlimb ischemia. CD31-positive cells are shown by arrows. Bar indicates 100 μm. D) Quantitative analysis of the number of CD31-positive endothelial cells in muscle fiber. E) Quantitative analysis of capillary density expressed as the number of CD31-positive cells per mm^2 28 days after the hindlimb ischemia. Data are expressed as means ± S.D. from 6 mice per group. *$P < 0.05$ vs. PBS treated mice (Student's *t*-test).

between PBS-treated mice and EPO (100 IU/kg)–treated mice ($P > 0.44$). Furthermore, the average number of CD31-positive cells per mm^2 in EPO (1000 IU/kg)–treated mice (398.9 ± 81.9) was significantly higher (about 2-fold) than that in PBS-treated mice (227.2 ± 12.4) (Fig. 1E).

EPO increases platelet adhesion to the endothelium in hind-limb microcirculation in the ischemic lesions

As demonstrated in Fig. 1, administration of EPO restored blood flow and increased tissue capillary density and platelet activity following hind-limb ischemia. These findings lead us to observe the hind-limb microcirculation and to examine the interaction of platelets with endothelium in ischemic tissues using in vivo microscopic studies. Adherent platelets were shown as fluorescent dots along the surface of the endothelial cells in the ischemic tissue (Fig. 2A). No adherent platelets in the microvessels were seen in non-ischemic tissue from mice treated with PBS or EPO (Fig. 2B). Intravital microscopic observation revealed that platelets directly bound to the endothelial cells, but were not indirectly attached through adherent leukocytes. Quantitative analysis revealed higher platelet-adherent microvascular density in EPO-treated mice than in PBS-treated mice (Fig. 2: C and D). In addition, platelet adhesion was observed at earlier time point, 2 days after the induction of ischemia, but there were no significant difference in the microvascular

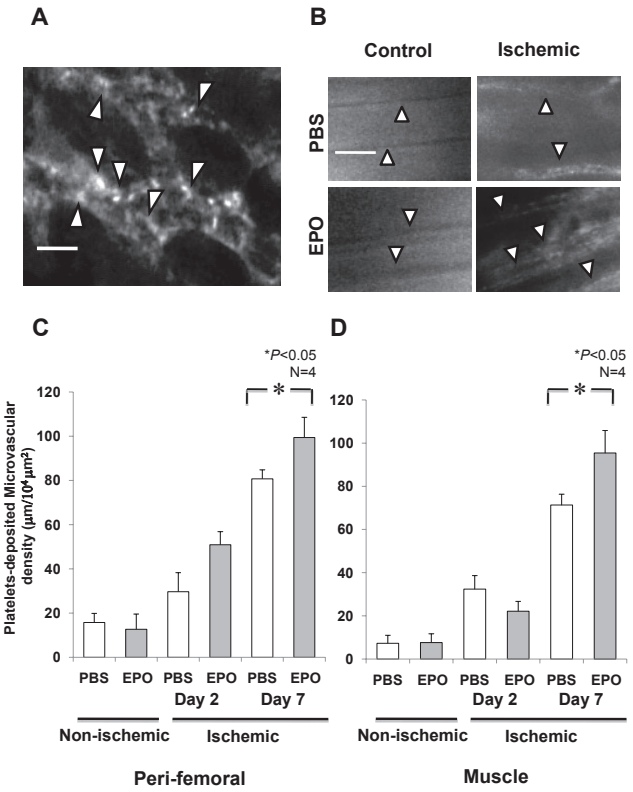

Fig. 2. Effect of EPO on hindlimb microcirculation following femoral artery ligation. A) Representative in vivo micrographs of hindlimb microcirculation 7 days after surgery. Note adherent platelets labeled with rhodamine 6G along the vessel wall. Bar indicates 15 μm. B) In vivo micrographs in the quadriceps muscle from control limb and ischemic limb. Arrow heads indicate capillaries. Bar indicates 30 μm. Quantification of platelet-deposited microvascular density in peri-femoral tissue (C) and in muscle tissue (D) from mice treated with EPO or PBS at 2 and 7 days after surgery. Data are expressed as means ± S.D. from 4 mice in each group. *$P < 0.05$ (Student's *t*-test).

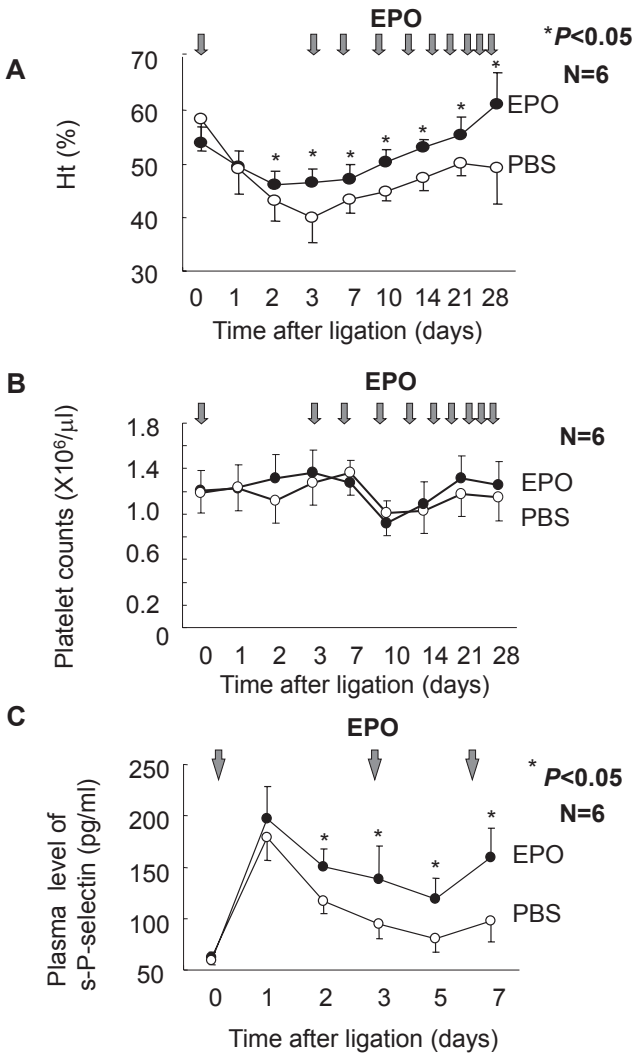

Fig. 3. Effects of EPO treatment on Ht, platelet counts, and plasma level of s-P-selectin. EPO treatment resulted in increased levels of Ht (A) and s-P-selectin (C), but not platelet counts (B) after the hind-limb ischemia. Plasma levels of s-P-selectin were determined using ELISA. Data are expressed as means ± S.D. from 6 mice per group. *$P < 0.05$ vs. PBS-treated mice (Student's *t*-test).

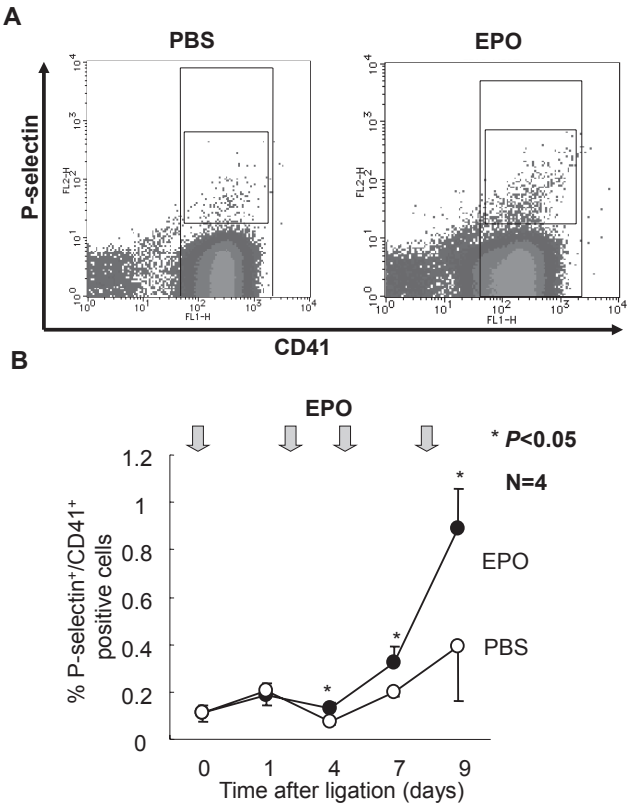

Fig. 4. Effect of EPO on expression of P-selectin$^+$/CD41$^+$ platelets following femoral artery ligation. P-selectin$^+$/CD41$^+$ platelets were determined as described in Materials and Methods. A) Representative flow cytometry images in PBS-treated mice and EPO-treated mice 7 days after the hind-limb ischemia. B) Time course of changes in the percentage of P-selectin$^+$/CD41$^+$ platelets in peripheral blood of EPO-treated mice and PBS-treated mice following femoral artery ligation. Data are expressed as means ± S.D. from 4 mice per group. *$P < 0.05$ vs. PBS-treated mice (Student's *t*-test).

density (Fig. 2: C and D).

Effect of EPO on levels of Ht, platelet count, and platelet activity

The levels of Ht in EPO-treated mice were higher than those in PBS-treated mice, at all time points studied except day 1 ($P < 0.05$) (Fig. 3A). Additionally, there was no significant difference in platelet counts between EPO-treated mice and controls (Fig. 3B). The results suggested that EPO had no effect on the production of platelets.

To determine whether EPO affects platelet activity, plasma levels of s-P-selectin were measured. Although hind-limb ischemia caused increases in s-P-selectin levels, the levels in EPO-treated mice were greater than those in PBS-treated mice at 2, 3, 5, and 7 days after surgery (Fig. 3C). Flow cytometric analysis demonstrated that the percentage of P-selectin–positive platelets in EPO-treated mice at 4, 7, and 9 days after surgery was greater than that in PBS-treated mice (Fig. 4: A and B). These results indicated that EPO enhanced platelet activity after femoral artery ligation.

Expression of EPOR on endothelial cells and platelets

Analysis in confocal laser microscopy on EPOR in the ischemic hind limb revealed that the endothelial cells were positively stained (Fig. 5A). By contrast, FACS analysis indicated that the positive fraction of EPOR in the circulating platelets estimated by CD41 expression was quite small before and after ligation (Fig. 5B).

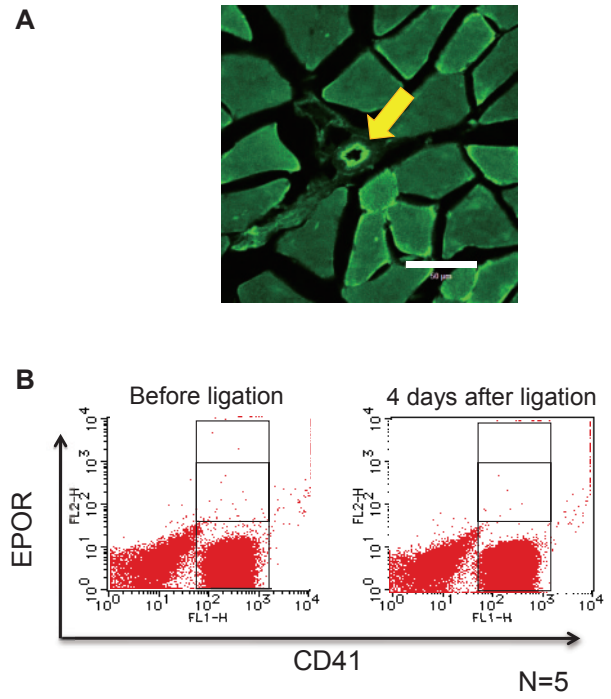

Fig. 5. Expressions of EPO-R on endothelial cells and platelets. A) A typical result in immunofluorescence. The tissue samples from ischemic hind limb were isolated 1 day after ligation. The sample sections were observed using a confocal scanning laser microscope. Bar indicates 50 μm. B) Results from FACS analysis. The blood samples were obtained before and 4 days after ligation. FACS analysis using CD41 antibody and EPOR antibody was performed as described in the text.

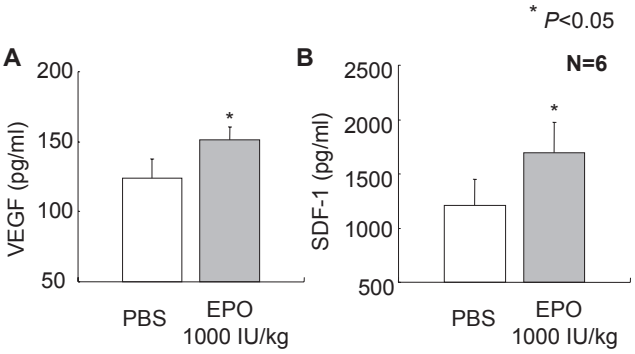

Fig. 6. Effect of EPO on levels of VEGF and of SDF-1 following femoral artery ligation. Plasma levels of VEGF (A) and SDF-1 (B) 3 days after induction of hind-limb ischemia were determined by ELISA. Data are expressed as means ± S.D. from 6 mice per group. *$P < 0.05$ vs. PBS-treated mice (Student's *t*-test).

Plasma levels of VEGF and SDF-1 after EPO administration

We determined plasma levels of VEGF (Fig. 6A) and SDF-1 (Fig. 6B) 3 days after the induction of hind-limb ischemia. In EPO-treated mice, both VEGF (151.6 ± 9.0 pg/ml) and SDF-1 (1690 ± 280 pg/ml) were significantly

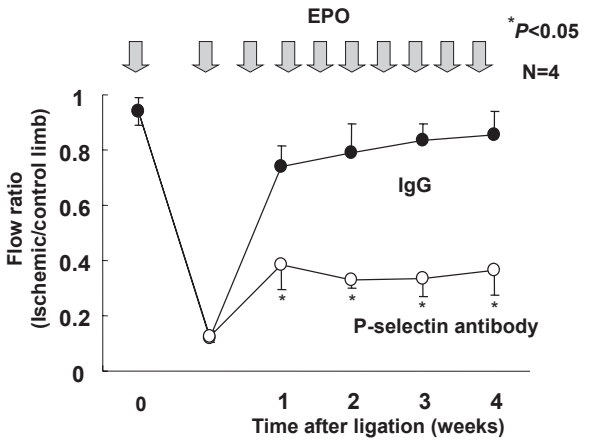

Fig. 7. Effect of anti–P-selectin antibody on EPO-induced blood flow recovery from hindlimb ischemia. EPO was intraperitoneally administered at the indicated time points. P-selectin antibody or vehicle (IgG) was treated (i.p.) every day after induction of hindlimb ischemia. Blood flow ratio was assessed by laser Doppler scanning before and at intervals after surgery. Data are expressed as means ± S.D. from 4 mice in each group. *$P < 0.05$ vs. IgG-treated mice (Student's *t*-test).

increased when compared with those in PBS-treated mice (VEGF: 123.8 ± 13.4 pg/ml and SDF-1: 1208 ± 240 pg/ml, respectively).

P-selectin–mediated platelet adhesion to the endothelium as a key event of enhancement of angiogenesis

Figure 7 shows the effect of P-selectin–neutralizing antibody on EPO-induced recovery of blood flow in response to acute hind-limb ischemia. P-selectin antibody–treated mice exhibited delayed blood flow recovery (0.36 ± 0.09) as compared to IgG-treated mice (0.85 ± 0.08) (Fig. 7).

Daily topical injection of P-selectin–neutralizing antibody markedly reduced plasma levels of VEGF (96 ± 11 pg/ml) and SDF-1 (543 ± 58 pg/ml) at 3 days after surgery compared with IgG treatment (VEGF: 158 ± 3 pg/ml and SDF-1: 1780 ± 250 pg/ml, respectively) (Fig. 8: A and B). These results suggested that EPO released VEGF and SDF-1 from activated platelets, resulting in enhanced the blood flow in ischemic tissue. To examine whether EPO-induced platelet adhesion to the vessels is dependent on P-selectin, the hind-limb microcirculation following femoral artery ligation was observed. The numbers of the microvessels to which platelets adhered in tissues in the vicinity of the ligated femoral artery (Fig. 8C) and the quadriceps muscle (Fig. 8D) were lower in anti–P-selectin antibody–treated mice than those in vehicle-treated mice. Quantitative analysis showed decreased platelet-deposited microvascular density in anti–P-selectin antibody–treated mice when compared with vehicle-treated mice (Fig. 8: E and F).

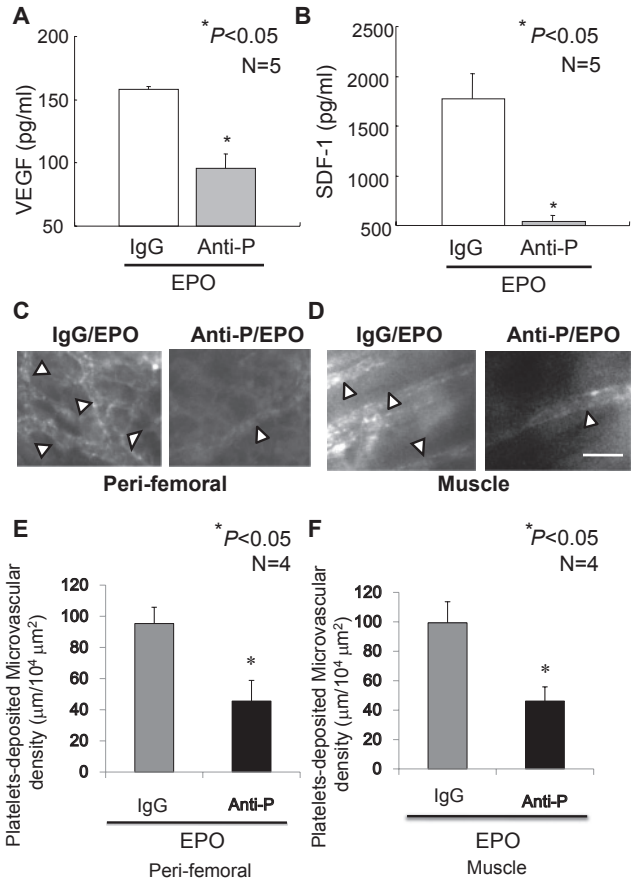

Fig. 8. Effects of anti–P-selectin antibody on plasma levels of VEGF and SDF-1 and hindlimb microcirculation following the induction of hindlimb ischemia in mice treated with EPO. Anti–P-selectin antibody or control IgG was administered (i.p.) to mice every day after surgery. EPO (1000 IU/kg) was administered (i.p.) three times per week. Plasma levels of VEGF (A) and SDF-1 (B) 3 days after the induction of hindlimb ischemia were determined with ELISA. Data are expressed as means ± S.D. from 5 mice in each group. *$P < 0.05$ vs. IgG-treated mice (Student's *t*-test). C and D) In vivo micrographs of hindlimb microcirculation in mice treated with IgG / EPO (C) or with anti–P-selectin antibody / EPO 7 days after surgery. Note adherent platelets labeled with rhodamine 6G along the vessel wall (C and D). Arrow heads indicate capillaries and arrows indicate venules. Bar indicates 30 μm. E and F) Quantification of platelet-deposited microvascular density in peri-femoral tissue (E) and in muscle tissue (F) from mice treated with IgG / EPO or with anti–P-selectin antibody / EPO. Data are expressed as means ± S.D. from 4 mice in each group. *$P < 0.05$ vs. control IgG-treated mice (Student's *t*-test).

Discussion

The present study revealed that EPO enhanced angiogenesis seen during ischemia in the hind-limb model and that P-selectin–mediated platelet adhesion to the endothelial cells in the ischemic lesions was critical for this enhancement (Figs. 1 and 7). Although it was previously reported that EPO enhanced angiogenesis in response to hind-limb ischemia (1), its precise mechanism of action was not fully elucidated. EPO is a 30.4-kDa protein that is produced and secreted from the kidney in response to anemia and hypobaric hypoxia. Binding of EPO to its receptor (EPO-R) on bone marrow erythroid progenitor cells results in the stimulation of red blood cell production. However, the biological effects of recombinant EPO therapy extend beyond the stimulation of erythropoiesis. The discovery that the EPO-R is expressed on vascular endothelial cells suggests that the vasculature may be a biological target of EPO. Indeed, several studies have now demonstrated that the protective effect of EPO administration involves the activation of the protein kinase B / Akt pathway that can protect cells from apoptosis. We showed here that EPO enhanced the recovery from ischemia via up-regulation of angiogenesis. In our preliminary experiment, we found that administration of EPO increased plasma levels of VEGF and SDF-1. We hypothesized that since platelets are known to be rich with cytokines or chemokines, such as VEFG and SDF-1, platelets may become a source of these molecules. Analysis by confocal laser microscopy on EPOR in the ischemic hind limb revealed that the endothelial cells were positively stained (Fig. 5A). By contrast, FACS analysis indicated that the positive fraction of EPOR in the circulating platelets estimated by CD41 expression was quite small (Fig. 5B). These suggested that besides direct action of EPO on the endothelial cells, platelet-mediated machinery may involve the enhancement of angiogenesis.

To reveal these issues, we used intravital microscopy (12). We selected the doses of EPO (100 and 1000 IU/kg) following the previous reports (13, 14). It was reported that the effect of hematopoiesis by these doses of EPO was dose-dependent in mice (13, 14), although a higher dose of EPO was necessary to induce enough hematopoiesis in mice when compared with humans. To see a sufficient response of EPO, we tested 1,000 IU/kg of EPO in the present study. We previously reported that P-selectin–mediated interaction between the platelets and the vascular endothelial cells was critical for the development of atherosclerosis in apoE-deficient mice (12). In the present study, we identified that P-selectin antibody blocked EPO-induced enhancement of angiogenesis and adhesion of platelets to the endothelial cells under exposure to hypoxia. These changes were accompanied with reductions in the plasma levels of VEGF and SDF-1 levels (Fig. 7: A and B), suggesting that these platelets may be sources of the plasma VEGF and SDF-1. Endothelial cells during hind-limb ischemia also could express P-selectin, suggesting that this molecule was important in vivo. Since P-selectin mediates the rolling of monocytes and the rolling is a prerequisite for extrava-

sation into the inflamed tissue, the possibility that P-selectin could be involved in the interaction of monocytes with endothelial cells during hind-limb ischemia can not be ruled out. Indeed, P-selectin–deficient mice exhibit impaired angiogenesis in response to hind-limb ischemia partly through inhibited recruitment of monocytes into the ischemic tissue (16).

When we observed microcirculation precisely, at 7 days after the induction of ischemia when revascularization began to take place, platelets adhesion to the microvessel walls did not disturb the blood flow because the size of the adhering platelets along the wall was not large enough to plug the lumen of the microvessels. In contrast, when platelets accumulate in the exposed subendothelium as a result of vascular injury, in which the endothelium was completely removed from the basement membrane, platelet aggregates become large enough to obstruct the microvasculature (17). Thus, the platelet adhesion without occlusion, as seen in the present study, may be a requisite for revascularization or tissue repair when platelets adhered to the endothelium in the process of angiogenesis (4, 18). It was reported that platelets preferentially adhere to the newly formed vessels in Matrigel in a skin chamber angiogenesis assay (18). It may be interesting to know the involvement of PSGL-1, a ligand of P-selectin in this EPO-induced enhancement of recovery from the ischemia. However, it was reported that PSGL-1 was expressed predominantly on the leukocytes and mainly mediated the rolling of leukocytes such as neutrophils (19). By contrast, P-selectin was present on the activated platelets and endothelial cells. As we observed in the present study, the increased interaction between the endothelial cells and the platelets was confirmed after EPO administration, but not that between the endothelial cells and the leukocytes, under intravital microscopy. These implied that the P-selectin–mediated interaction between the endothelial cells and the platelets was critical in the present study. SDF-1 is a potent chemokine and SDF-1 expression in ischemic tissues is crucial for mobilization of hematopoietic stem and progenitor cells, enhancing angiogenesis and tissue recovery (20, 21). Activated platelets release SDF-1 and thereby recruit VEGFR1+ hematopoietic progenitor cells (HPCs) in addition to VEGFR2 + EPCs to neoangiogenic sites within 7 days after the ligation of femoral artery (22 – 24). VEGF recruits endothelial progenitors through enhancement of SDF-1 expression (22, 25). In addition, EPO has been reported to increase mobilization of EPCs (CD34 + VEGFR2+) from bone marrow 7 days after the induction of hind-limb ischemia (1). These findings suggest that EPO exerts its pro-angiogenic effect by facilitating the recruitment of bone marrow–derived pro-angiogenic cells through enhancement of VEGF and SDF-1 expression during the early phase of revascularization in response to hind-limb ischemia, although a precise and targeted strategy to identify the involvement of VEGF and SDF-1 is still needed.

In conclusion, increased platelet activity and platelet adhesion appear to be critical for EPO-induced neovascularization from hind-limb ischemia and for release of VEGF and SDF-1. P-selectin signaling on the platelet deposits may be relevant to inducing the releases of both angiogenic factor VEGF and SDF-1. Administration of EPO may be useful for supporting revascularization of ischemic tissue.

Acknowledgments

We thank Michiko Ogino, Keiko Nakamigawa, Mieko Hamano, and Osamu Katsumata for their technical assistance; Dr. Leon Bax at the Kitasato Clinical Research Center for statistical advice; and Dr. Patrick Hughes for correcting English in the manuscript.

References

1 Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood. 2003;102:1340–1346.
2 Peterson TE, Katusic ZS. EPO tecting the endothelium. Br J Pharmacol. 2007;150:823–825.
3 Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. J Clin Invest. 2005;115:3378–3384.
4 Pinedo HM, Verheul HM, D'Amato RJ, Folkman J. Involvement of platelet in tumor angiogenesis? Lancet. 1998;352:1775–1777.
5 Pipili-Synetos E, Papadimitriou E, Maragoudakis ME. Evidence that platelets promote tube formation by endothelial cells on matrigel. Br J Pharmacol. 1998;125:1252–1257.
6 Amano H, Hackett NR, Rafii S, Crystal RG. Thrombopoietin gene transfer-mediated enhancement of angiogenic response to acute ischemia. Circ Res. 2005;97:337–345.
7 Iba O, Mastubara H, Nozawa Y, Fujiyama S, Amano K, Mori Y, et al. Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemia limbs. Circulation. 2002;106:2019–2025.
8 Kopp HG, Rafii S. Thrombopoietic cells and the bone marrow vascular niche. Ann N Y Acad Sci. 2007;106:175–179.
9 Kirkeby A, Torup L, Bochsen L, Kjalke M, Abel K, Theilgaard-Monch K, et al. High-dose erythropoietin alters platelet reactivity and bleeding time in rodents in contrast to the neuroprotective variant carbamyl-erythropoietin (CEPO). Thromb Haemost. 2008;99:720–728.
10 Stohlawetz PJ, Dzirlo L, Hergovich N, Lackner E, Mensik C, Eichler HG, et al. Effects of erythropoietin on platelet reactivity and thrombopoiesis in humans. Blood. 2000;95:2983–2989.
11 Sayinalp N, Erdem Y, Ilhami Ozcebe O, Büyükaşik Y, Cağlar S, Dündar SV. Recombinant human erythropoietin increases platelet aggregation in chronic hemodialysis patients. Thromb Res. 1998;90:195–198.
12 Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, et al. Roles of thromboxane A(2) and prostacyclin

in the development of atherosclerosis in apoE-deficient mice. J Clin Invest. 2004;114:784–794.
13 Heeschen C, Aicher A, Lehmann R, Fichtischerer S, Vasa M, Urbich C, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood. 2003;102: 1340–1346.
14 Bleuel H, Hoffmann R, Kaufmann B, Neubert P, Ochlich PP, Schaumann W. Kinetics of subcutaneous versus intraveneous epoetin-beta in dogs, rats and mice. Pharmacology. 1996;52: 329–338.
15 d'Uscio LV, Smith LA, Santhanam AV, Richardson D, Nath KA, Katusic ZS. Essential role of endothelial nitric oxide synthase in vascular effects of erythropoietin. Hypertension. 2007;49: 1142–1148.
16 Egami K, Murohara T, Aoki M, Matsuishi T. Ischemia-induced angiogenesis: role of inflammatory response mediated by P-selectin. J Leukoc Biol. 2006;79:971–976.
17 Langer H, Gawaz M. Platelets in regenerative medicine. Basic Res Cardiol. 2008;103:299–307.
18 Kisucka J, Butterfield CE, Duda DG, Eichenberger SC, Saffaripour S, Ware J, et al. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. Proc Natl Acad Sci U S A. 2006;103:855–860.
19 Xu T, Zhang L, Geng ZH, Wang HB, Wang JT, Chen M, et al. P-selectin cross-links PSGL-1 and enhances neutrophil adhesion to fibrinogen and ICAM-1 in a Src kinase-dependent, but GPCR-independent mechanism. Cell Adh Migr. 2007;1:115–123.
20 Petit I, Jin D, Rafii S. The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. Trends Immunol. 2007;28:299–307.
21 Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation. 2003;107:1322–1328.
22 Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, et al. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med. 2006;12:557–567.
23 Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Chadburn A, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med. 2001;7:1194–1202.
24 Massberg S, Konrad I, Schurgzinger K, Lorenz M, Schneider S, Zohlnhoefer D, et al. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. J Exp Med. 2006;203:1221–1233.
25 Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell. 2006;124:175–189.