Ischemia-induced angiogenesis: role of inflammatory response mediated by P-selectin

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Abstract: P-selectin is a 140-kDa glycoprotein expressed on endothelial cells and platelets. P-selectin mediates the tethering and rolling of leukocytes along the endothelium, an early step of leukocyte extravasation. Although inflammation is a requisite process for ischemia-induced angiogenesis, little is known regarding the role of P-selectin in angiogenesis in the setting of tissue ischemia. We examined whether ischemia-induced angiogenesis is altered in P-selectin knockout (P-selectin$^{-/-}$) mice. Angiogenesis was evaluated in a surgically induced hind-limb ischemia model using laser Doppler blood flowmetry (LDBF) and histological capillary density (CD). After left hind-limb ischemia, the ischemic/normal limb LDBF ratio was persistently lower in P-selectin$^{-/-}$ mice compared with wild-type (WT) mice. CD was also significantly lower in P-selectin$^{-/-}$ mice than in WT mice on Postoperative Day 14. Fewer numbers of total CD45+ inflammatory leukocytes infiltrated into the ischemic tissues in P-selectin$^{-/-}$ mice than in WT mice, and immunohistochemical analysis revealed the number of infiltrated leukocytes expressing vascular endothelial growth factor was also decreased in P-selectin$^{-/-}$ mice. P-selectin mRNA expression was augmented after hind-limb ischemia in WT mice. In conclusion, P-selectin may play an important role in ischemia-induced angiogenesis by promoting early inflammatory mononuclear cell infiltration. P-selectin would become one possible target molecule for modulating inflammatory angiogenesis. *J. Leukoc. Biol.* 79: 971–976; 2006.

Key Words: *peripheral circulation · growth factor · animal model · human disease*

INTRODUCTION

Postnatal neovascularization is regulated by various angiogenic growth factors, cytokines, bone marrow-derived progenitor cells, inflammatory leukocytes, extracellular matrices, and vasoactive substances [1–3]. It has been shown that leukocyte infiltration early after ischemia is an important trigger for ischemia-induced angiogenesis, as inflammatory cells can release various angiogenic cytokines including vascular endothelial growth factor (VEGF) [4, 5].

P-selectin is a 140-kDa glycoprotein, one of three selectin class cell adhesion molecules, and is expressed on activated endothelial cells and platelets [6–8]. P-selectin is normally stored in the Weibel Palade bodies of endothelial cells or in α-granules of platelets and is translocated rapidly to the cell surface upon activation with inflammatory mediators [6]. P-selectin mediates tethering and rolling of leukocytes along the endothelium, an early requisite step for subsequent leukocyte extravasation [6–8].

Considering the fact that inflammation is important for ischemia-induced angiogenesis, P-selectin could play a role in angiogenesis after ischemia, as P-selectin supports an early stage of leukocyte migration. However, little is known as to whether P-selectin plays a role in the process of ischemia-induced angiogenesis in vivo.

Accordingly, taking advantage of using genetically modified P-selectin knockout (P-selectin$^{-/-}$) mice [8], we examined whether P-selectin plays a role in ischemia-induced angiogenesis in vivo. We used a well-established mouse model of angiogenesis with unilateral hind-limb ischemia [9], and we focused on whether inflammatory response is altered after induction of tissue ischemia in P-selectin$^{-/-}$ mice.

MATERIALS AND METHODS

Animals

P-selectin$^{-/-}$ mice with a C57/BL6J background were generated as described previously [8] and were obtained from The Jackson Laboratory (Bar Harbor, ME). Control wild-type (WT) C57/BL6J strain mice were purchased from the Clea Japan. Male P-selectin$^{-/-}$ mice and WT mice were used at the age of 8–12 weeks.

Mouse model of unilateral hind-limb ischemia

The study protocols were approved by the Institutional Animal Care and Use Committee. We used a mouse model of angiogenesis, in which the entire left femoral artery and vein were excised surgically [4, 9]. When hind-limb ischemia was induced, new blood vessels grew into the ischemic limb. We prepared this model in P-selectin$^{-/-}$ mice and WT mice to determine whether

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ischemia-induced angiogenesis was affected by the deficiency of P-selectin. In brief, mice were subjected to unilateral hind-limb ischemia under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally). Before surgery, body weight and systemic arterial blood pressure (SBP) were determined. SBP was determined using a tail-cuff pressure analysis system (TK370C, Unicom, Tokyo, Japan) in the conscious state. Capillary angiogenesis and hind-limb blood flow were examined by the methods below.

Laser Doppler blood flow analysis

We measured the ratio of the ischemic (left)/normal (right) hind-limb blood flow by laser Doppler blood flowmetry (LDBF; moorLDI, Moor Instruments, Wilmington, DE) [4, 9]. At seven predetermined time-points (before surgery and at Postoperative Days 1, 3, 7, 14, 21, and 28), we performed laser-beam scanning over the legs and feet. The average LDBF of the ischemic and nonischemic hind limbs was then computed. To minimize variations as a result of ambient light, blood flow was expressed as the ischemic (left)/normal (right) hind-limb LDBF ratio [4, 9].

Determination of the capillary density (CD)

Five animals in each group were killed on Postoperative Day 14 with an overdose of sodium pentobarbital. Medial thigh adductor muscles of ischemic and nonischemic limbs were harvested, and some tissues were fixed in methanol, and the others were processed for frozen sections. Methanol-fixed tissues were embedded in paraffin, and 5 μm-thick sections were prepared. The fixed sections were stained with a rat anti-mouse anti-CD31 monoclonal antibody (mAb). Frozen sections were stained with alkaline phosphatase (AP) to analyze tissue CD [10]. Five microscopic fields from the three muscle samples of each animal were selected randomly for the capillary counts.

Evaluation of inflammatory cell infiltration

Five animals in each group were killed on Postoperative Day 3 with an overdose of sodium pentobarbital. Medial thigh adductor muscles of ischemic (left) limbs were harvested and fixed in methanol. Tissues were embedded in paraffin, and 5 μm-thick sections were prepared. The sections were stained for hematoxylin and eosin (H&E) to detect inflammatory cells. Sections were also stained using a rat anti-mouse CD45 mAb to detect infiltrated leukocytes [4].

Infiltrated leukocytes, especially macrophages, release angiogenic cytokines including VEGF, which promote angiogenesis [4]. We thus examined VEGF protein using a double-immunofluorescence staining. Cryostat sections with 5 μm in thickness were mounted on silicone-coated slides. They were incubated overnight at 4°C with an anti-mouse VEGF mAb (Santa Cruz Biotechnology, CA) and with an anti-mouse macrophage mAb (F4/80, Dako, Carpinteria, CA) in a moist chamber. The slides were then incubated for 30 min at 37°C with a fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G (IgG) antibody (Zymed Laboratories, San Francisco, CA) to detect VEGF. Then, they were incubated further for 30 min at 37°C with a phycoerythrin-conjugated anti-rat IgG (Serotec, Oxford, UK) to detect macrophages [11]. Slides were examined and photographed under fluorescence microscopy (Diaphot 300, Nikon, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Using semiquantitative RT-PCR analysis, we examined whether expression of P-selectin and E-selectin mRNA was altered in the ischemic hind-limb tissues of WT mice. Total RNA was extracted from ischemic hind-limb tissues using guanidium isothiocyanate-phenol chloroform solution (TRIzol reagent, Invitrogen, Carlsbad, CA) before ischemic surgery and at Days 1, 3, 7, and 14 after the surgery (n=3 at each time-point). Isolated, total RNA was quantified by measuring absorption at 260/280 nm and subjected to RT-PCR analysis [12]. Total RNA was reverse-transcribed using oligo dT primers and RNase H RT (Superscript II, Invitrogen) with 1 μg total RNA per sample [12]. The primer set for detecting mouse P-selectin mRNA was for (sense) 5′-CCTGGCAAGTGGAATGATGA-3′ and for (antisense) 5′-AAGCTGCAGACTGACTGGTA-3′. Mouse E-selectin was for (sense) 5′-AGAACTGGGCTCCAGGTGAA-3′ and for (antisense) 5′-GGACTTGTAGGTGAATTCTCC-3′. Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was for (sense) 5′-ACCACAGTCCATGCCATCAC-3′ and for (antisense) 5′-TCCACCACCCTGTTGCTGTA-3′. RT-PCR of P-selectin, E-selectin, and GAPDH was expected to yield PCR products, sized 820 base pairs (bp; GenBank NM011347), 1084 bp (GenBank NM011345), and 452 bp (GenBank M32599), respectively.

Measurements of tissue monocyte chemoattractant protein-1 (MCP-1) and VEGF levels by enzyme-linked immunosorbent assay (ELISA)

As infiltration of macrophages is associated with the expression of MCP-1, we determined tissue levels of MCP-1 by ELISA. Ischemic skeletal muscles were isolated, homogenized, and centrifuged for 15 min at 2500 *g* at 4°C. Supernatant was then recovered, and MCP-1 levels were determined using a mouse MCP-1 ELISA kit (Quantikine M, R&D Systems, Minneapolis, MN) [11]. As infiltrated macrophages release an angiogenic cytokine VEGF, we also determined the tissue VEGF levels using a mouse VEGF ELISA kit (Quantikine M, R&D Systems) [11].

Statistical analysis

Data are expressed as mean ± SE. Differences between the two groups were analyzed by unpaired Student's *t*-test. *P* values <0.05 were considered to be statistically significant.

RESULTS

LDBF

Figure 1A shows representative images of LDBF, which revealed a progressive recovery of the ischemic/normal hind-limb LDBF ratio during 28 days after induction of hind-limb ischemia in WT mice. However, the blood flow in the ischemic leg was reduced in P-selectin$^{-/-}$ mice. Figure 1B summarizes the calculated LDBF ratio. After the operative induction of left hind-limb ischemia, the ratio decreased to almost 0.1 in all mice, showing no differences of the ratio between the two groups. Thus, the severity of induced hind-limb ischemia was comparable between the two groups. The ratios of the ischemic to normal blood flow at Postoperative Days 3, 7, 14, 21, and 28 were significantly smaller in P-selectin$^{-/-}$ mice than in WT mice.

Tissue CD

Representative photomicrographs of histological sections stained with anti-CD31 mAb or AP are shown in **Figure 2A**. CD31^{+}or AP-positive capillaries were identified in transverse sections of skeletal muscle tissues on Postoperative Day 14. The number of capillary vessels was reduced significantly in P-selectin$^{-/-}$ mice in slides stained with CD31 mAb or AP (Fig. 2A). Figure 2B shows quantitative data of the number of capillary vessels (per ×400 field) counted using AP-stained histological sections, which revealed that the CD was significantly lower in P-selectin$^{-/-}$ mice than in WT mice ($P<0.001$). There was no difference in the CD in contralateral, nonischemic skeletal muscles (data not shown).

Inflammatory leukocyte infiltration

On Days 3 and 7 after ischemia, we examined inflammatory leukocyte infiltration by H&E staining and CD45 immunostaining. Representative photomicrographs of histological sections and summarized quantitative data are shown in **Figure 3, A** and **B**. There were significantly smaller numbers of

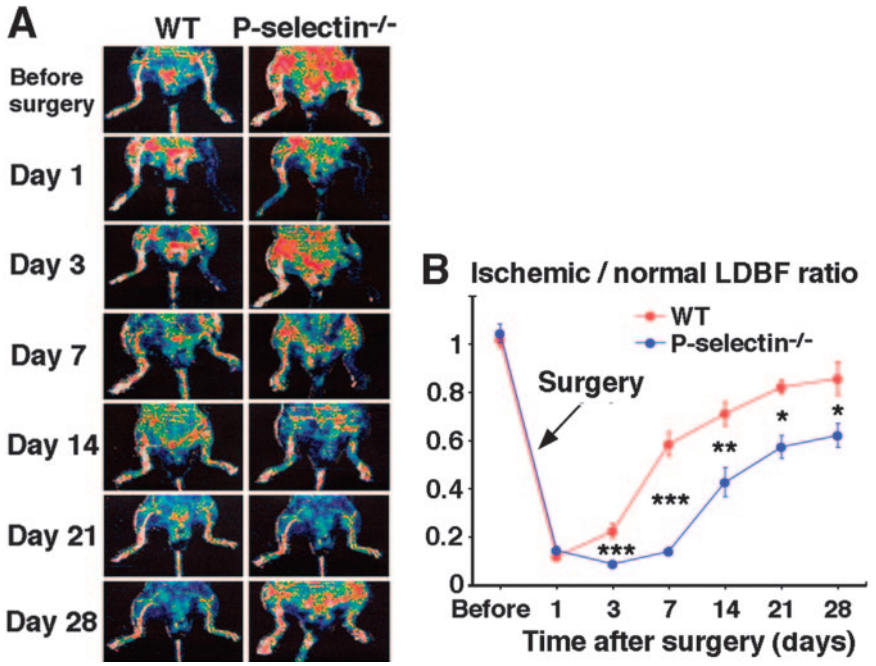

Fig. 1. (A) The recovery of the ischemic hind-limb blood flow was reduced in P-selectin$^{-/-}$ mice. (B) The LDBF ratio was significantly smaller in P-selectin$^{-/-}$ mice than in WT mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inflammatory leukocytes in the ischemic tissues in P-selectin$^{-/-}$ mice than in WT mice on Days 3 and 7.

P-selectin and E-selectin mRNA expression

We examined whether P-selectin and E-selectin mRNA were up-regulated after the induction of hind-limb ischemia. Semi-quantitative RT-PCR analysis (**Fig. 4**) showed that P-selectin mRNA expression was up-regulated in WT mouse hind-limb tissues after ischemia. In contrast, E-selectin mRNA expression was only slightly elevated after the induction of tissue ischemia (Fig. 4).

Immunohistochemical localization of VEGF and macrophages

Inflammatory cell infiltration supports angiogenesis by releasing various angiogenic cytokines. We thus examined whether infiltrated macrophages release VEGF using two-color immunofluorescence histochemical staining. **Figure 5** shows that infiltrated F4/80-positive macrophages were costained with anti-VEGF mAb, indicating that infiltrated macrophages produce VEGF. Furthermore, the number of infiltrated, VEGF-positive macrophages was lower in P-selectin$^{-/-}$ mice than in WT mice.

Tissue VEGF and MCP-1 analysis by ELISA

Macrophage infiltration depends largely on tissue expression of MCP-1, and infiltrated macrophages release VEGF protein. We thus examined tissue levels of VEGF and MCP-1. **Figure 6** indicates that tissue concentrations of VEGF and MCP-1, as assessed by ELISA, were significantly lower in P-selectin$^{-/-}$ mice than in WT mice.

DISCUSSION

Angiogenesis in tissues exposed to severe ischemia is a major survival mechanism against hypoxic tissue injury [13, 14]. The present study clearly demonstrates that ischemia-induced angiogenesis is significantly impaired in P-selectin$^{-/-}$ mice, and its mechanism likely involves the reduction of tissue inflammatory responses and angiogenic cytokine release.

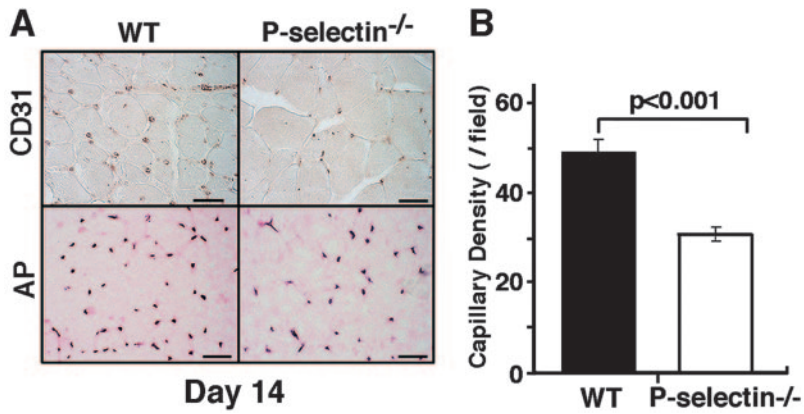

Fig. 2. (A) CD31^{+}or AP-positive capillaries were identified in skeletal muscle tissues on Day 14. The number of capillary vessels was reduced in P-selectin$^{-/-}$ mice in CD31 and AP staining. Original bars = 50 μm. (B) The calculated CD was significantly lower in P-selectin$^{-/-}$ mice than in WT mice.

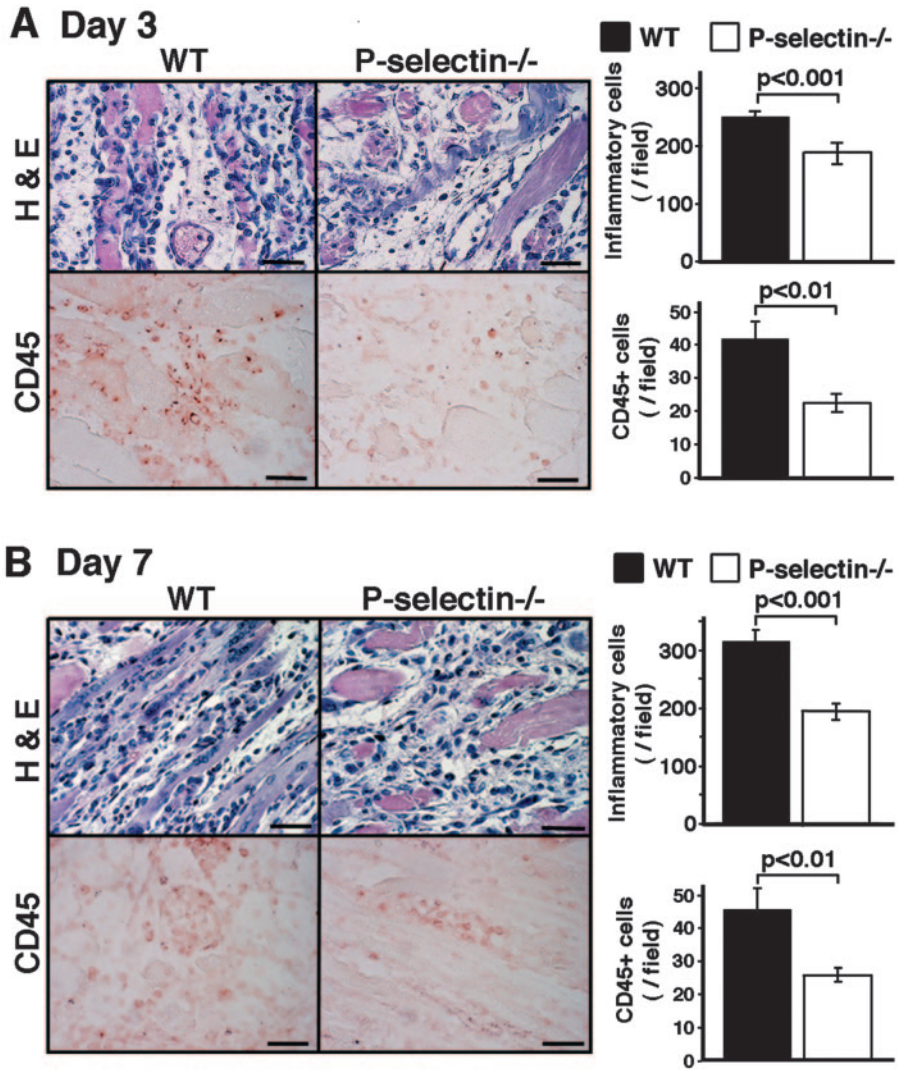

Fig. 3. (A and B) Representative photomicrographs of histological sections and summarized quantitative data revealed that there were lower numbers of inflammatory leukocytes infiltrated in ischemic tissues in P-selectin$^{-/-}$ mice than in WT mice on Days 3 (A) and 7 (B) after ischemia.

During an early inflammatory process such as tissue ischemia, P-selectin mediates leukocyte rolling on the endothelium. This process is followed by a firm adhesion of leukocytes to the endothelium and subsequent extravasation. Among three selectin class cell adhesion molecules (P-, E-, and L-selectins), P-selectin has unique biological characteristics. First, P-selectin is normally stored in intracellular granules of quiescent endothelial cells and platelets; however, upon activation with various inflammatory mediators, P-selectin translocates rapidly from their stores to the cell surface. Second, P-selectin expression is usually limited to the inflammatory sites. Therefore, P-selectin has major proinflammatory roles [6–8]. Consistently, the functional blockade of P-selectin has been shown to protect against various inflammatory tissue injuries including myocardial ischemia-reperfusion injury and inflammatory lung disease [15, 16].

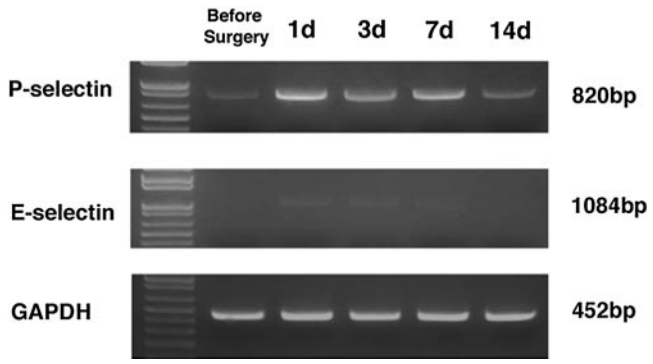

Fig. 4. Semiquantitative RT-PCR showed that P-selectin mRNA expression was up-regulated in WT mouse hind-limb tissues after the induction of ischemia. In contrast, E-selectin mRNA expression was only slightly enhanced after the induction of tissue ischemia.

Although leukocyte infiltration early after tissue ischemia may be injurious in one aspect, this response could also be a requisite for the tissue repair process, which includes reparatory angiogenesis, fibrosis, and thus, wound healing. Infiltrated leukocytes can release various angiogenic cytokines, which stimulate angiogenesis [2]. We recently showed that inflammation occurring after tissue ischemia played an important role in subsequent angiogenesis [4]. In the present study, leukocyte infiltration at the early phase of ischemia was impaired significantly in P-selectin$^{-/-}$ mice compared with WT mice. We further confirmed that P-selectin mRNA expression was indeed

up-regulated in limb tissue after ischemia in WT mice. As E-selectin might share overlapping roles with P-selectin in terms of leukocyte rolling on the endothelium [17], we also examined the expression of E-selectin mRNA. However, E-selectin mRNA expression was only slightly elevated after tissue ischemia (Fig. 4). These results are consistent with our current finding that the deficiency in the P-selectin gene alone sufficiently suppressed leukocyte infiltration after hind-limb ischemia.

Dual immunofluorescence staining revealed that infiltrated macrophages released VEGF protein, and the number of VEGF-positive macrophages was reduced in P-selectin$^{-/-}$ mice compared with WT mice. Consistently, the contents of VEGF protein in tissue homogenates were significantly lower in P-selectin$^{-/-}$ mice than in WT mice by ELISA. Taken together, P-selectin plays an important role in inflammatory leukocyte infiltration and their angiogenic cytokine production, which would be relevant for angiogenesis in the setting of tissue ischemia.

In the present study, the level of MCP-1 was also reduced in ischemic tissues in P-selectin$^{-/-}$ mice. This finding is interesting, as MCP-1 is another potent, arteriogenic chemokine by recruiting monocytes to the inflammatory tissues [18]. It is well known that not only endothelium but also activated platelets express P-selectin [19]. Weyrich and co-workers [20] showed previously that activated platelets bind to monocytes via P-selectin, and they stimulate monocytes to release MCP-1. Thus, it is conceivable that platelet P-selectin-mediated activation of local monocytes might be diminished in P-selectin$^{-/-}$ mice, which resulted in the decrease in the tissue MCP-1 levels.

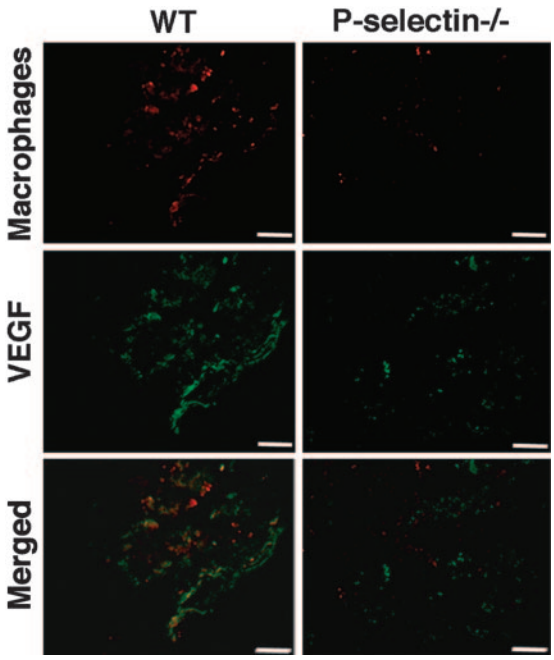

Fig. 5. Two-color immunofluorescence staining showed infiltrated macrophages (red) and VEGF protein (green) in adjacent ischemic skeletal muscle sections. In the merged pictures, there are some overlaps between macrophage infiltration and VEGF protein expression, suggesting that these infiltrated macrophages can produce and release VEGF protein. The number of macrophage infiltration was greater in WT mice than in P-selectin$^{-/-}$ mice. Original bars = 50 μm.

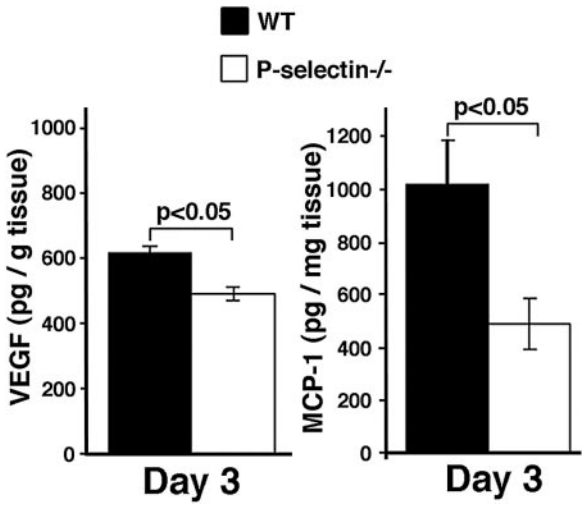

Fig. 6. Tissue concentrations of VEGF and MCP-1 proteins quantified by ELISA were significantly lower in P-selectin$^{-/-}$ mice than in WT mice, suggesting smaller secretion of VEGF and MCP-1 in the ischemic tissues in P-selectin$^{-/-}$ mice than in WT mice.

Study limitations

In a previous study, Hartwell and co-workers [21] demonstrated, using a mouse corneal micropocket assay, that angiogenesis was not inhibited in P-selectin$^{-/-}$ or E-selectin$^{-/-}$ mice. The precise reason for the discrepancy of the results between the study by Hartwell and co-workers [21] and the current study is unclear. However, the difference could be well explained. The normal cornea is free from pre-existing vessels and contains no blood. Therefore, angiogenesis in the cornea may depend mainly on endothelial sprouting, migration, and proliferation but depend to a lesser extent on inflammatory leukocyte infiltration. Conversely, in the hind-limb ischemia model, there is a massive infiltration of leukocytes from the pre-existing, inflamed vessels. Therefore, the leukocyte infiltration could be more important for the ischemia-induced angiogenesis as compared with the corneal angiogenesis. It is thus reasonable that the ischemia-induced angiogenesis but not the corneal angiogenesis was inhibited in P-selectin$^{-/-}$ mice in vivo [21].

In the present study, the role of P-selectin on activated platelets is still unclear. Activated platelets may also release VEGF, which further promotes angiogenesis [22]. Therefore, P-selectin expressed on activated platelets might play some roles in angiogenesis in vivo. The specific role of P-selectin expressed on platelets warrants further investigation in the future.

In summary, we have first demonstrated that ischemia-induced angiogenesis is significantly impaired in P-selectin$^{-/-}$ mice. Our results suggest that inflammatory response early after tissue ischemia is a requisite process for the ischemia-induced angiogenesis. P-selectin would become one possible target molecule for modulating inflammatory angiogenesis.

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