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Background—Although potential participation of bone marrow-derived circulating endothelial progenitor cells (EPCs) to neoangiogenesis has been proposed, the precise molecular mechanisms of EPC recruitment to vascular endothelium has not been fully elucidated.

Methods and Results—Peripheral blood mononuclear cells were isolated from healthy volunteers and cultured for 7 days to obtain EPCs. Tumor necrosis factor-α–activated human umbilical vein endothelial cells (HUVECs) supported significantly more rolling and adhesion of EPCs compared with inactivated HUVEC monolayer. Pretreatment of activated HUVEC with an adhesion-blocking mAb to E-selectin significantly reduced EPCs adhesion to HUVECs. When HUVECs were transduced with a recombinant adenovirus of E-selectin (AdRSVE-sel) or that of β-galactosidase (AdRSVLacZ), E-selectin-transduced but not LacZ-transduced HUVECs exhibited significantly more EPC rolling as well as adhesion. Further, effect of AdRSVE-sel or AdRSVLacZ was examined in mouse hind limb ischemic model. AdRSVE-sel–transduced mice showed significantly less limb necrosis and higher laser Doppler ratio when compared with AdRSVLacZ-transduced mice. Interestingly, blood flow recovery of ischemic limb observed in AdRSVE-sel–transduced mice was more prominent when combined with EPC administration compared with that of AdRSVLacZ-transduced mice.

Conclusions—Endothelial E-selectin plays a crucial role in EPC–endothelial interaction in vitro. The importance of E-selectin was also confirmed in vivo even in the absence of exogenous EPC. These data provide molecular background for novel cell-based therapy for ischemic atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2007;27:512-518.)**

Key Words: angiogenesis ■ adhesion molecule ■ endothelial cell

Recent studies have provided ample evidence that vasculogenesis, the formation of new blood vessels by differentiation of endothelial cells from their precursors, may occur after embryonic development. The identification of endothelial progenitor cells (EPCs) in circulating blood in postnatal subjects and their contribution to the formation of new blood vessels[1–11] has drawn attention to their potential for therapeutic application. Although a study that used an ischemic animal model[12] and carefully conducted clinical[13] studies have documented the efficacy of the introduction of EPCs in ischemic tissues, the molecular mechanisms responsible for EPC recruitment into mature preexisting blood vessels are not fully understood. Several growth factors, including vascular endothelial growth factor,[2] stem cell growth factor,[9] and granulocyte-macrophage colony-stimulating factor,[3] have been reported to be involved in the regulation of endothelial differentiation and migration to form functional vessels. However, the interaction between circulating EPCs and mature blood endothelial cells, presumably a critical biological event during the initial phase of vasculogenesis, has not been extensively studied. In some studies, the number of exogenous EPCs present in the ischemic tissue has been reported to be not as much at 28 days as compared with 1 day after injury, suggesting that not only EPC but also the soluble factor they secreted or expressed may also be important to facilitate neovasculogeneisis.[14] To form properly assembled blood vessels, EPC orchestrate complex cell–cell adhesive interactions by coordinated actions of adhesion molecules and their ligands expressed on both EPC and preexisting endothelial cells.

E-selectin, a member of selectin family of adhesion molecules, is expressed primarily on activated vascular endothelium and facilitates rolling and adhesion of neutrophils, monocytes, and a subset of T lymphocytes.[15] In addition to its role in acute and chronic inflammation, E-selectin has been shown to participate in ischemia/reperfusion injury in hu-

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mans[16] and, more importantly, E-selectin and its ligands have been reported to be involved in angiogenesis.[17] In the present study, we used a well-defined parallel plate flow chamber apparatus and demonstrated that E-selectin expressed on activated endothelial cells plays a major role in rolling and adhesion of EPC in vitro. That initial observation was confirmed by use of a recombinant adenoviral vector of E-selectin. Our in vitro results were confirmed in vivo by using an ischemic hind limb model. Our findings indicate that E-selectin is a major adhesion receptor on preexisting endothelial cells that function to initiate postnatal neoangiogenesis. Further, overexpression of E-selectin may be useful for therapeutic usage of EPCs, the current availability of which limits potential clinical outcomes.

Methods

EPC Culture and Reagents

Peripheral blood mononuclear cells from human volunteers were plated on human fibronectin coated (Sigma) culture dishes and maintained in endothelial cell basal medium-2 (Clonetics) supplemented with 5% fetal bovine serum, human vascular endothelial growth factor-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid, as previously described.[1,18] After 4 days of culture, nonadherent cells were removed by washing with phosphate-buffered saline, then cultured 3 more days before use in a simulated flow assay.

Flow-Cytometric Analysis of EPCs

After 7 days of culture, adherent cells were detached and incubated first with the indicated primary antibodies on ice, then washed twice with RPMI 1640 containing 5% fetal bovine serum. Next, they were incubated with a fluorescein isothiocyanate-labeled goat anti-mouse antibody. Fluorescence was analyzed using a fluorescence-activated cell sorter Caliber (Beckton-Dickinson).

Adenovirus Transduction of HUVECs

In some experiments, after a 24-hour incubation on coverslips, HUVECs were transduced using a recombinant adenovirus of human E-selectin (AdRSVE-sel)[19] or that of β-galactosidase (AdRSVLacZ)[20] at an MOI of 100, 72 hours before use in an EPC flow assay, as described previously.[21]

Adhesion Assay Under Laminar Flow

Apparatus Design

The parallel-plate flow chamber used was previously described in detail.[22] Briefly, the chamber was composed of 2 aluminum steel plates separated by a 200-μm-thick silastic gasket, and the flow channel was formed by removal of a 2.5×15-mm rectangular section from the gasket. Predetermined levels of flow were applied to the HUVEC monolayer by drawing the perfusion medium (D-PBS containing 0.2% human serum albumin) through the channel with a syringe pump (model 44; Harvard Apparatus). A plastic heating plate (Tokai Hit Co) was mounted on the stage of an inverted microscope (IX50; Olympus) to maintain the temperature at 37°C. The channel flow could then be approximated as a 2-dimensional fully developed laminar flow with a simple parabolic velocity profile.

Experimental Application

HUVECs were isolated for use in the flow assay, and then after 2 or 3 passages were plated onto 22-mm fibronectin coated glass cover slips and subjected to the flow chamber. Flow assay endothelial monolayers on coverslips were stimulated with tumor necrosis factor-α (10 ng/mL) and positioned in the flow chamber, which was mounted on an inverted microscope. Each monolayer was perfused for 3 minutes with perfusion medium and examined carefully to verify that it was confluent. Then, EPCs were diluted in the perfusion medium to 10^5 cells/mL and drawn through the chamber at a controlled flow rate to generate a calculated wall shear stress of 1.0 dyne/cm^2 for 10 minutes. The entire period of perfusion was recorded on videotape using a digital video recorder containing a time generator. Captured images were then transferred to a computer for image analysis to determine the number of rolling and adherent EPC in 5 to 10 randomly selected 20× microscopic fields for each experiment. Cells were considered to be adherent after 10 seconds of stable contact with the monolayer. Rolling EPCs were easily recognized, as their velocity was much slower (up to 80 μm/second) than that of free-flowing cells.

In some experiments, the following antibodies were used to block EPC adhesion to HUVEC monolayers: mouse anti-E-selectin mAb (7A9), mouse anti-intercellular adhesion molecule-1 (ICAM-1) mAb (Hu5/3), mouse anti-vascular cell adhesion molecule (VCAM)-1 (BBIG-V), mouse anti-sLx (KM-93), mouse anti-PSGL-1 (KPL-1), mouse anti CD18 (MEM48).

Mouse Hind limb Ischemia Model

All animal procedures were performed in accordance with guidelines provided by the Tokyo Medical and Dental University Institutional Animal Care and Use Committee. Athymic nude mice (Crea, Japan), 8 to 9 weeks old and 17 to 20 grams in weight, were used. First, AdRSVE-sel or AdRSVLacZ (at 1x10^{10} pfu/limb) was injected into a lower limb of 19 mice. Seventy-two hours later, each mouse was anesthetized intraperitoneally with 160 mg/kg of pentobarbital for operative ligation of a femoral artery and vein, as previously described.[4,13] In some experiments, EPCs at 1×10^5, 20% of the optimal number reported previously,[4,13] in 100 μL of endothelial cell basal medium-2 media without any growth factors were injected into the ischemic limb muscle of the mice immediately after the ligation procedure. To determine the fate of the transplanted EPCs, 4 mice from each group received EPCs marked with fluorescent carbocyanine DiI dye (Molecular Probes), as previously described.[23] In brief, suspended EPCs were washed with phosphate-buffered saline and incubated with DiI at a concentration of 2.5 μg/mL of phosphate-buffered saline for 5 minutes at 37°C, then 15 minutes at 4°C. After 2 washings in phosphate-buffered saline, the cells were suspended in endothelial cell basal medium-2 medium. Further, 30 minutes before these mice were euthanized, they received an intravenous injection of 50 μg of Bandeiraea simplicifolia lectin 1 (BS-1) conjugated with fluorescein isothiocyanate (Vector Laboratories).

Physiological Assessment of Animals

Laser Doppler perfusion imaging (Moor Instruments) was used to record blood flow 28 days after surgery, as previously described.[23] In the digital color-coded images, the red hue indicated regions with maximum perfusion, whereas medium perfusion levels were shown as yellow and low levels as blue. The resulting images also displayed absolute values in readable units. For quantification, the ratio of readable units between ischemic and nonischemic hind limbs was determined.

Histological Assessment of Mouse Tissues

Vascular density in sections taken from the ischemic hind limbs was evaluated at the microvascular level using a light microscope. Tissue sections from the lower calf muscles of ischemic limbs were harvested on day 28, then muscle samples were embedded in OCT compound (Miles), snap-frozen in liquid nitrogen (LN_2), and cut into 5-μm-thick sections. A total of 20 different fields were randomly selected and the number of capillaries was counted at 40× magnification. Immunohistochemical analysis of human E-selectin was carried out using anti human E-selectin mAb (7A9) as a primary antibody. MOM immunodetection kit (Vector Laboratories) was used to specifically localize mouse primary antibody (7A9) on mouse tissues following manufacturer's instruction.

Statistical Analysis

All results were expressed as mean±SEM. Statistical significance was evaluated using a paired Scheffé *t* test or one-way ANOVA with

Tukey post hoc test. A value of $P<0.05$ was considered to denote statistical significance.

Results

Characterization of Human EPCs

In preliminary experiments, we confirmed the surface expression of markers for endothelial cells (CD34, CD31, and vascular endothelial growth factor receptor 2) in EPCs after 7 days of culture (data not shown). We also examined the uptake of DiI acetylated LDL and colony formation by EPCs to confirm that they had an endothelial lineage (data not shown). Based on those basic characterizations, we used EPCs after 7 days of culture in the following experiments.

Activation With Tumor Necrosis Factor-α Increased EPC Rolling and Adhesion to Vascular Endothelium Under Physiological Flow Conditions

To monitor the adhesive interactions of EPCs, we utilized a parallel plate flow chamber system, which was initially designed to investigate leukocyte–endothelial interactions under physiological conditions.[22,24] When EPCs were perfused over a monolayer of inactivated HUVECs, virtually no interaction was observed. In contrast, when HUVEC monolayers were activated with tumor necrosis factor-α, a cytokine that is strongly upregulated during inflammation, including that in the ischemic vasculature, EPCs showed significant rolling and adhesion (Figure 1A and 1B). A time-kinetic analysis revealed that maximum induction of EPC rolling and adhesion occurred at 4 hours of stimulation with tumor necrosis factor-α. Similarly, rolling and adhesion of EPCs were observed when HUVECs were stimulated with IL-1β (10 U/mL, data not shown). Interestingly, when EPCs were stimulated with tumor necrosis factorα, EPC adhesion to HUVECs was not induced (Figure 1C).

To determine the adhesion molecules responsible for the observed interaction with EPCs HUVEC monolayers were pre-incubated with function blocking antibodies against E-selectin (7A9), ICAM-1 (Hu5/3), and VCAM-1 (BBIGV1), and the results were compared with those with a binding control mAb (w6/32). As shown in Figure 2A, pretreatment with 7A9, but not the others, significantly blocked the rolling and adhesion of EPCs to HUVECs, suggesting a potential role for E-selectin in the recruitment of EPCs to HUVEC monolayers.[22]

SLx and PSGL-1 Were Not Involved in Initial Adhesive Interactions of EPCs to HUVEC Monolayers

Next, we investigated the potential ligands expressed on EPCs that interact with E-selectin in HUVEC monolayers. As previously reported, sialyl Lewis X (sLx), a carbohydrate has been known to bind to E-selectin.[25] Further, P-selectin glycoprotein 1 (PSGL-1), a putative ligand for P-selectin, has also been shown to bind E-selectin.[26] Although these antibodies were confirmed to inhibit monocyte adhesion to activated HUVECs (sLx, 37.05% reduction; PSGL-1 35.1% reduction; Figure 2B upper right column), the EPC endothelial interaction was not blocked by antibodies against sLx or PSGL-1 (Figure 2B).

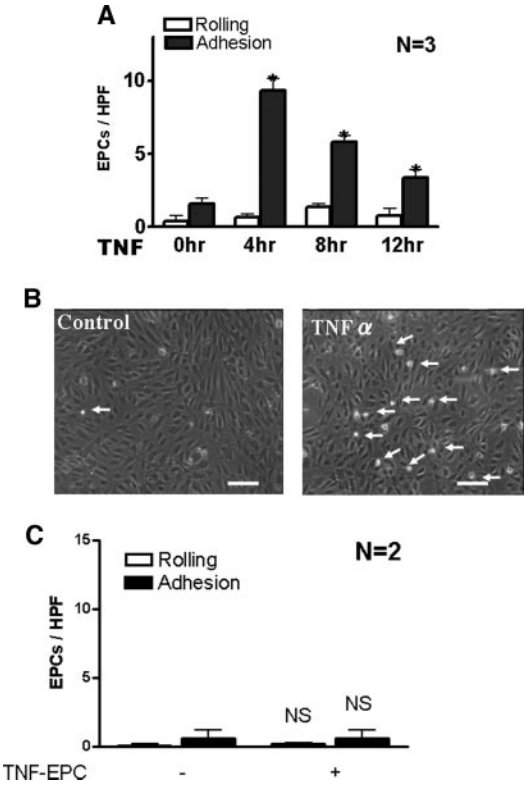

Figure 1. Adhesive interactions of EPCs on activated HUVEC monolayers under flow conditions. A, EPCs at 10 000/mL (after 7 days in culture) were perfused over HUVEC monolayers activated with tumor necrosis factor-α (5 ng/mL) for the indicated times, as described in Methods. The average number of rolling (white bar) and adherent (black bar) cells in 10 randomly selected microscopic fields are shown as representative samples of 4 similar experiments. *$P<0.05$ vs tumor necrosis factor-α (−). B, Representative micrographs of adhesive interactions of EPCs (arrow) on HUVEC monolayers, activated with tumor necrosis factor-α (5 ng/mL) 4 hours, are shown. Scale bar=100 μm. C, EPCs at 10 000/mL (after 7 days in culture) were activated with tumor necrosis factor-α (5 ng/mL) for 4 hours and perfused over HUVEC monolayers as described in Methods. The average number of rolling (white bar) and adherent (black bar) cells in 10 randomly selected microscopic fields are shown as representative samples of 2 similar experiments.

Overexpression of E-Selectin Without Cytokine Stimulation Induced EPC Recruitment to HUVEC Monolayer

To verify the involvement of E-selectin in the adhesive interaction of EPCs with a HUVEC monolayer, we transduced HUVECs with a recombinant adenoviral vector of E-selectin (AdRSVE-sel) and compared the results to those transduced with a recombinant adenovirus expressing β-galactosidase (AdRSVLacZ). Both adenoviral vectors were transduced at an MOI of 100 and an adhesion assay under flow was performed 72 hours after transduction. As shown in Figure 3, AdRSVE-sel–transduced HUVECs supported significantly greater levels of rolling and adhesion as compared with those transduced with AdRSVLacZ. Further, the inhibition of EPC adhesion seen after pretreatment with 7A9 confirmed the involvement of E-selectin in HUVECs transduced with the adenoviral vectors.

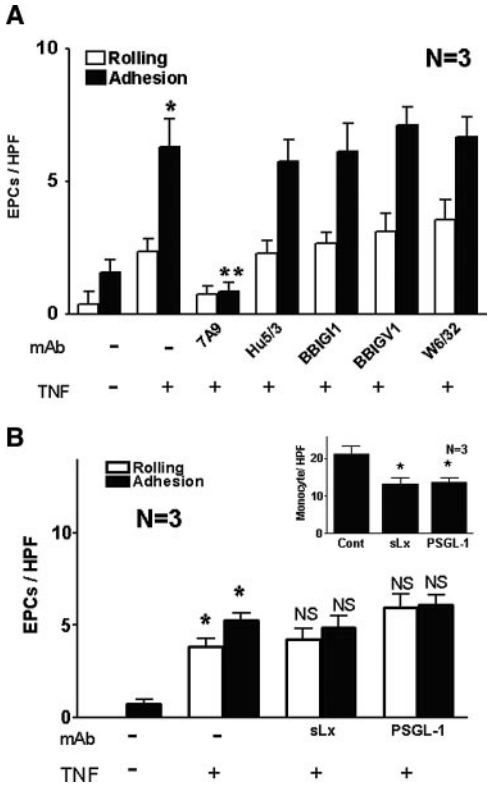

Figure 2. A, Characterization of adhesion of EPCs to HUVEC monolayers under flow conditions. Activated HUVEC monolayers (tumor necrosis factor-α, 5 ng/mL, 4 hours) were pre-incubated with mAbs against E-selectin (7A9), ICAM-1 (Hu5/3, BBIGI1), VCAM-1 (BBIGV1), and the control (W6/32, HLA-A,B,C) at 10 μg/mL for 20 minutes before an adhesion assay as detailed in Methods. Data were shown as representative samples of 3 similar experiments. *$P<0.01$ vs tumor necrosis factor-α (−); **$P<0.001$ vs tumor necrosis factor-α (+) mAb (−). B, Activated HUVEC monolayers (tumor necrosis factor-α, 5 ng/mL, 4 hours) were pre-incubated with mAbs against sLx (KM-93), PSGL-1 (KPL-1), at 10 μg/mL for 20 minutes before an adhesion assay using EPCs, as detailed in Methods. Data were shown as representative samples of 3 similar experiments. *$P<0.03$ vs tumor necrosis factor-α (−). N.S. vs no antibody. Right upper columns, Inhibitory effects of these antibodies were confirmed adhesion assay using human monocyte to HUVEC monolayers under flow conditions. Activated HUVEC monolayers (tumor necrosis factor-α, 5 ng/mL, 4 hours) were pre-incubated with mAbs against sLx (KM-93), PSGL-1 (KPL-1), at 10 μg/mL for 20 minutes beforeo an adhesion assay. Data were shown as representative samples of 3 similar experiments. *$P<0.03$ vs control.

Role of E-Selectin in EPC Transplantation in Murine Ischemic Hind limb Model

The potential role of E-selectin in neovascularization in vivo was investigated using a murine hind limb ischemia model. An intramuscular administration of AdRSVE-sel or AdRSVLacZ (1×10^{10}pfu/limb) was injected into the left limb muscle 72 hours before femoral artery excitation and neovascularization was examined without EPC injection. As shown in Figure 5A, AdRSVE-sel was able to potentiate neovascularization even in the absence of exogenous EPCs. Perfusion ratio of ischemic limb was recovered up to 75% of noninjured contra lateral limb at 28 days after ischemia (Figure 4B). However, perfusion ratio of ischemic limb in AdRSVLacZ-transduced mice was much lower (38.94% of contra lateral limb; Figure 4B). To validate the

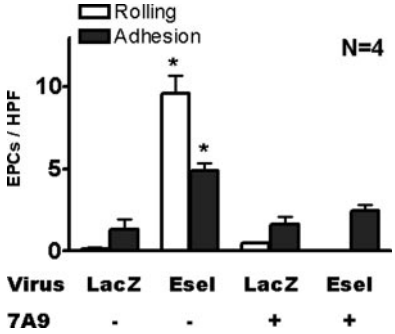

Figure 3. EPC adhesion assay using nonactivated HUVEC monolayers transduced with an E-selectin adenovirus (AdRSVE-sel) or control LacZ adenovirus (AdRSVLacZ). Each monolayer (1×10^5 cells per cover slip) was transduced with AdRSVE-sel or AdRSVLacZ at an MOI of 100 and an adhesion assay was performed 72 hours later, as described in Methods. In some experiments, adenovirus-transduced HUVEC monolayers were pre-incubated with an anti-E-selectin mAb (7A9) for 20 minutes before the assay. Representative data from 4 similar experiments are shown. *$P<0.03$ vs control.

contribution of endogenous progenitor cells in this process, immunohistochemical localization of CD34-positive cells at the site of ischemia was examined. As shown in (Figure 4C), clusters of CD34-positive cells were found only in AdRSVE-sel–transduced but not AdRSVLacZ-transduced mouse, suggesting their contribution to neovascularization. The expression of exogenous E-selectin was confirmed with a fluorescent immunohistochemical staining using anti human E-selectin mAb 7A9 (Figure 4D).

We further investigated a role of E-selectin in neovascularization with EPC transplantation. One day after operative excision of a left femoral artery from each mouse, EPC at 1×10^5 ($\approx$20% of the optimal number of EPCs used in previous studies, which was insufficient to induce optimal neovascularization[13]) were injected into each ischemic limb. Examination of hind limb perfusion by laser Doppler perfusion imaging was performed on days 0 and 28, and the significant reduction of perfusion seen on day 0 confirmed the effectiveness of our ischemic procedure (Figure 5A, middle column). On day 28, the ratio of ischemic to normal blood flow in mice injected with AdRSVE-sel was strikingly improved, as compared with those injected with AdRSVLacZ (Figure 5A, right column). The blood flow of AdRSVE-sel–injected animal was recovered to the level of baseline. This improvement in blood flow in AdRSVE-sel–injected animal was also validated in representative photos (Figure 5B). Enhanced neovascularization in mice overexpressing E-selectin after EPC transplantation resulted in a significant improvement of tissue salvage, despite frequent auto-amputation of the ischemic limb attributable to the inherent impairment of neovascularization in athymic nude mice. In mice that received AdRSVLacZ, limb salvage (score 0 in Figure 5C) was achieved in only 1 of 9 animals, whereas extensive limb necrosis (score 4 and 5) developed in 4 of the remaining 8. In contrast, successful limb salvage (score 0) was achieved in 5 of the 10 mice that received AdRSVE-sel and limb necrosis (score 4) was limited to 1 (Figure 5C). A histological examination on day 28 revealed that capillary density in mice transduced with AdRSVE-sel plus EPC

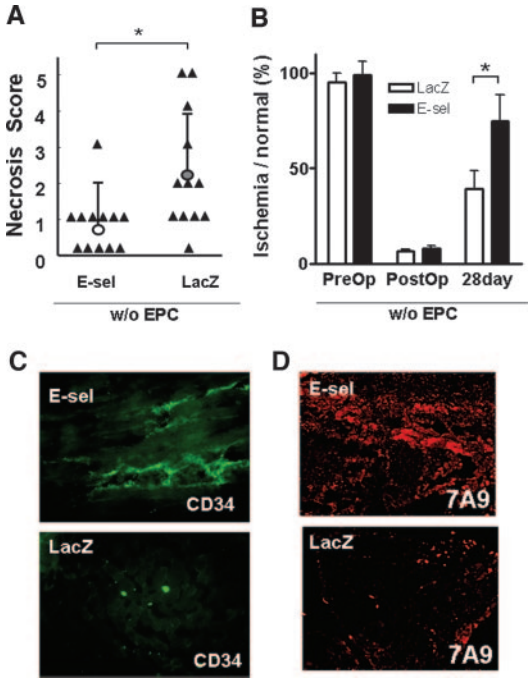

Figure 4. A, Evaluation of limb salvage on day 28, as described in Methods. Shown are the necrosis scores of mice transduced with AdRSVE-sel (E-sel, number of animals examined=12) or AdRSVLacZ (LacZ, number of animals examined=12). * $P<0.03$. B, The average rate of perfusion in each limb (ischemic/nonischemic) was calculated based on laser doppler perfusion imaging analysis before and after (28 days) the ischemic procedure in mice transduced with AdRSVE-sel (black column, E-sel) or AdRSVLacZ (white column, LacZ). Perfusion is expressed as ratio between the ischemic and non-ischemic limbs. Representative data from 3 similar experiments are shown. Each experiment incorporated of at least 5 animals per condition. *$P<0.01$ vs LacZ at 28 days. C, Representative fluorescence micrograph of ischemic limb muscle from athymic mouse transduced with AdRSVE-sel (E-sel) or AdRSVLacZ (LacZ). Tissue sections were stained using rat anti-mouse CD34 mAb followed by fluorescein isothiocyanate-labeled secondary antibodies to recognize CD34 positive murine cells. 200× magnification. D, Representative fluorescence micrograph of ischemic limb muscle from athymic mouse transduced with AdRSVE-sel (E-sel) or AdRSVLacZ (LacZ). Tissue sections were stained using mouse anti-human E-selectin mAb followed by fluorescein isothiocyanate-labeled secondary antibodies with MOM immunodetection kit as described in Methods. 200× magnification.

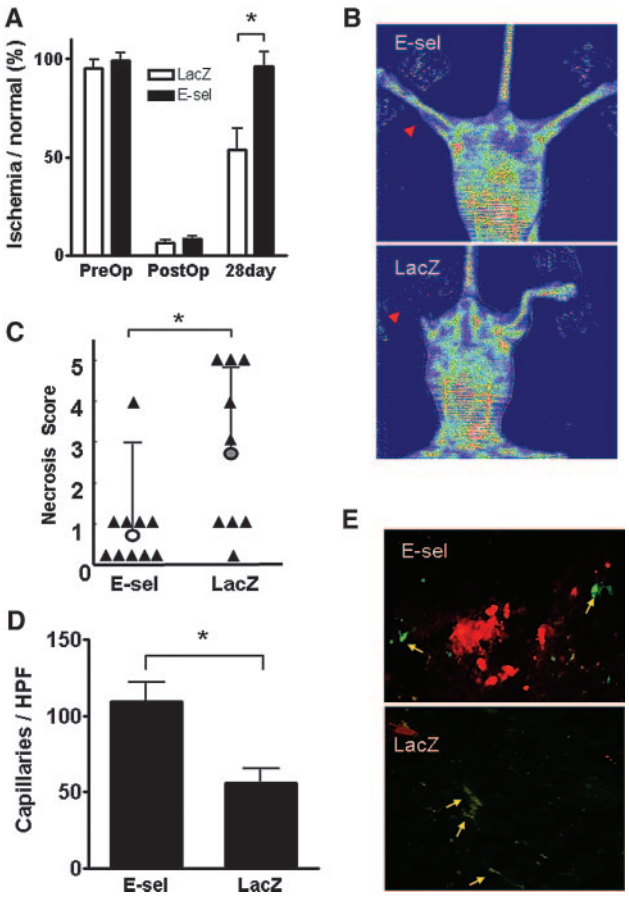

Figure 5. Contribution of human EPCs to neo-angiogenesis in hind limbs of ischemic mice. A, The average ratio of perfusion in each limb (ischemic/nonischemic) was calculated based on laser Doppler perfusion imaging analysis immediately before (PreOp) and after (PstOp) ischemic procedure and 28days after procedures (28 days). Perfusion is expressed as ratio between the ischemic and nonischemic limbs in mice transduced with AdRSVE-sel (black column, E-sel) or AdRSVLacZ (white column, LacZ). Representative data from 3 similar experiments are shown. Each experiment incorporated of at least 5 animals per condition. * $P<0.001$ vs LacZ at 28 days. B, Representative laser Doppler perfusion imaging image of AdRSVE-sel-transduced (E-sel) or AdRSV-LacZ–transduced (LacZ) mice taken 28 days after the ischemic procedure. Arrow head indicates the location where femoral artery was ligated. C, Evaluation of limb salvage on day 28, as described in Methods. Shown are the necrosis scores of mice transduced with AdRSVE-sel (E-sel, number of animals examined=10) or AdRSVLacZ (LacZ, number of animals examined=9). *$P<0.03$. D, Histological evidence of neovascularization was assessed by measuring capillary density. Representative data from 3 similar experiments are shown. Each experiment incorporated of at least 5 animals per condition. *$P<0.02$. E, Representative fluorescence micrograph of ischemic limb muscle from athymic mouse transduced with AdRSVE-sel. Red fluorescence indicates DiI labeling of transplanted EPC and green indicates BS-1 lectin, a marker of endothelial cells (yellow arrow heads). 200× magnification.

transplantation was significantly higher than in mice transduced with AdRSVLacZ and EPC transplantation (Figure 5D). To confirm the homing and incorporation of administered EPC in the sites of neovascularization, EPC labeled with fluorescent carbocyanine DiI dye were injected intramuscularly after excision of a left femoral artery. As shown in (Figure 5E), skeletal muscle sections prepared from the ischemic hind limbs 3 days after the mice received AdRSVE-sel exhibited an accumulation of EPCs (red cluster) in areas surrounding the injection site. Such accumulations of injected EPCs were not observed in sections from mice that received AdRSVLacZ (Figure 5E).

Discussion

Our results demonstrated a potential role of endothelial E-selectin in the recruitment of EPC to facilitate neovascularization in ischemic tissues in vitro and in vivo. The present findings also showed the molecular requirements for the initial interaction of EPC and mature pre-existing endothelium. After identification of EPCs in the circulation of human adults, numerous reports have indicated the potential importance and therapeutic application of these cells in vivo using animal models of ischemia.[1] Thus far, broad attention has

been given to angiogenic enzymes, growth factors, and their receptors; however, the direct interaction of these progenitor cells to form blood vessels also requires cell adhesion molecules.[27]

Although the mechanisms responsible for leukocyte–endothelial interaction have been hypothesized, the precise molecular mechanisms involved with the initial EPC–endothelial interactions have yet to be investigated thoroughly. We found that E-selectin expression is necessary and sufficient to capture circulating EPC to a mature endothelial monolayer. Our findings are in agreement with previous observations that have focused on leukocyte–endothelial interactions in vivo[15] and confirm the existence of similar adhesive properties in interacting vascular endothelial cells. The interaction of leukocytes to activated endothelial cells starts with the leukocytes rolling on the endothelial monolayer, which is primarily supported by the selectin family of adhesion molecules, after which they stably adhere via integrin and ICAM-1/VCAM-1 interactions. The present findings are the first to show that EPCs also follow the same adhesive interaction cascade. However, it is intriguing that mAbs against ICAM-1 and VCAM-1, adhesion molecules important for the stable adhesion of leukocytes in the presence of flow, failed to block the adhesion under our assay conditions. This result may imply the importance of other adhesion molecules, including novel ones, in this step of the adhesion cascade, which is different from what is known regarding leukocytes. In addition, the failure of antibodies against sLx or PSGL-1 to block the E-selectin dependent adhesion of EPCs suggests the role of an unknown ligand on the surface of EPCs and, considering its relatively broad ligand specificity,[28,29] the contribution of known ligands with E-selectin should not be neglected.

The potential role of E-selectin in angiogenesis has been suggested by the results of other studies,[30,31] whereas soluble E-selectin was shown to induce neovascularization in mouse corneas, as well as induce endothelial migration and tube formation in vitro.[32] As demonstrated by the present findings, overexpression of E-selectin is also necessary for circulating EPCs to adhere to a mature endothelial monolayer. To confirm that captured EPCs contribute to neovascularization, we performed in vivo experiments using hind limbs from a murine ischemic model. Our results showed that an administration of E-selectin adenovirus significantly enhanced capillary formation and perfusion, and reduced necrosis caused by ischemia, as compared with control LacZ-transduced mice. Moreover, overexpression of E-selectin alone, although less efficient than combination with EPCs, can potentiate neovascularization in ischemic hind limb model, suggesting that exogenous E-selectin may be able to capture murine endogenous progenitor cells, as shown in Figure 5C.

It is known that a low number of cells obtained from each donor limit the therapeutic application of EPCs, although several approaches have been proposed to overcome this limitation. Injections of granulocyte-macrophage colony-stimulating factor into the peripheral circulation have been reported to cause the migration of EPCs from bone marrow,[5] which might be a useful method to collect a large number of EPCs. In addition, the overexpression of vascular endothelial growth factor using gene transfer techniques[33] was also demonstrated to be an efficient method for enhancing angiogenesis in vivo.

As compared with those strategies that employed soluble factors, our approach, which used membrane-bound E-selectin, has several advantages, such as site-specific overexpression caused by a local injection of an E-selectin vector, and minimized unnecessary systemic effects. Considering its potential in neovascularization in the absence of exogenous EPCs, overexpression of E-selectin could be a promising approach to potentiate neovascularization.

In conclusion, the present results showed that an administration of E-selectin might be a promising novel technique to efficiently utilize EPCs in therapeutic neovascularization. Further, our data strongly indicate a primary role of E-selectin expressed on activated vascular endothelium for the recruitment of EPCs in vitro and in vivo.

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Disclosures

None.

References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
2. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J*. 1999;18:3964–3972.
3. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res*. 1999;85:221–228.
4. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T, Symes JF. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. *Ann Thorac Surg*. 2000;70:829–834.
5. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med*. 1999;5:434–438.
6. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K-i, Eguchi H, Onitsuka I, Matsui K, Imaizumi T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest*. 2000; 105:1527–1536.
7. Shi Q, Rafii S, Wu MH-D, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP. Evidence for Circulating Bone Marrow-Derived Endothelial Cells. *Blood*. 1998;92:362–367.
8. Hatzopoulos A, Folkman J, Vasile E, Eiselen G, Rosenberg R. Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development*. 1998;125:1457–1468.
9. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood*. 2000;95:3106–3112.
10. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF. Endothelial Cells of Hematopoietic Origin Make a

Significant Contribution to Adult Blood Vessel Formation. *Circ Res*. 2000;87:728–730.
11. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE. Contribution of Monocytes/Macrophages to Compensatory Neovascularization : The Drilling of Metalloelastase-Positive Tunnels in Ischemic Myocardium. *Circ Res*. 2000;87:378–384.
12. Folkman J. Therapeutic Angiogenesis in Ischemic Limbs. *Circulation*. 1998;97:1108–1110.
13. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization [In Process Citation]. *Proc Natl Acad Sci U S A*. 2000;97:3422–3427.
14. Collinson DJ, R. D. Therapeutic angiogenesis in peripheral arterial disease: can biotechnology produce an effective collateral circulation? *Eur J Vasc Endovasc Surg*. 2004;28:9–23.
15. Yoshida M, Westlin WF, Wang N, Ingber DE, Rosenzweig A, Resnick N, Gimbrone MA, Jr. Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. *J Cell Biol*. 1996;133:445–455.
16. Bevilacqua MP, Nelson RM, Mannori G, Cecconi O. Endothelial-leukocyte adhesion molecules in human disease. *Annu Rev Med*. 1994;45:361–378.
17. Aoki M, Kanamori M, Yudoh K, Ohmori K, Yasuda T, Kimura T. Effects of vascular endothelial growth factor and E-selectin on angiogenesis in the murine metastatic RCT sarcoma. *Tumour Biol*. 2001;22:239–246.
18. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation*. 2002;105:3017–3024.
19. Yoshida M, Szente BE, Kiely JM, Rosenzweig A, Gimbrone MA, Jr. Phosphorylation of the cytoplasmic domain of E-selectin is regulated during leukocyte-endothelial adhesion. *J Immunol*. 1998;161:933–941.
20. Ishii H, Yoshida M, Rosenzweig A, Gimbrone MA, Jr., Yasukochi Y, Numano F. Adenoviral transduction of human E-selectin into isolated, perfused, rat aortic segments: an ex vivo model for studying leukocyte-endothelial interactions. *J Leukoc Biol*. 2000;68:687–692.
21. Luscinskas F, Brock A, Arnaout M, Gimbrone M, Jr. Endothelial-leukocyte adhesion molecule-1-dependent and leukocyte (CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. *J Immunol*. 1989;142:2257–2263.
22. Gerszten RE, Luscinskas FW, Ding HT, Dichek DA, Stoolman LM, Gimbrone MA, Jr., Rosenzweig A. Adhesion of memory lymphocytes to vascular cell adhesion molecule-1-transduced human vascular endothelial cells under simulated physiological flow conditions in vitro. *Circ Res*. 1996;79:1205–1215.
23. Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation*. 2002;105:732–738.
24. Luscinskas FW, Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Jr., Luster AD, Rosenzweig A. C-C and C-X-C chemokines trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Ann N Y Acad Sci*. 2000;902:288–293.
25. Kansas G. Selectins and their ligands: current concepts and controversies. *Blood*. 1996;88:3259–3287.
26. Zollner TM, Asadullah K. Selectin and selectin ligand binding: a bittersweet attraction. *J Clin Invest*. 2003;112:980–983.
27. Nguyen M, Strubel NA, Bischoff J. A role for sialyl Lewis-X/A glycoconjugates in capillary morphogenesis. *Nature*. 1993;365:267–269.
28. Hammel M, Weitz-Schmidt G, Krause A, Moll T, Vestweber D, Zerwes HG, Hallmann R. Species-specific and conserved epitopes on mouse and human E-selectin important for leukocyte adhesion. *Exp Cell Res*. 2001;269:266–274.
29. Larsen GR, Sako D, Ahern TJ, Shaffer M, Erban J, Sajer SA, Gibson RM, Wagner DD, Furie BC, Furie B. P-selectin and E-selectin. Distinct but overlapping leukocyte ligand specificities. *J Biol Chem*. 1992;267:11104–11110.
30. Kraling BM, Razon MJ, Boon LM, Zurakowski D, Seachord C, Darveau RP, Mulliken JB, Corless CL, Bischoff J. E-selectin is present in proliferating endothelial cells in human hemangiomas. *Am J Pathol*. 1996;148:1181–1191.
31. Bischoff J, Brasel C, Kraling B, Vranovska K. E-selectin is upregulated in proliferating endothelial cells in vitro. *Microcirculation*. 1997;4:279–287.
32. Kenyon B, Voest E, Chen C, Flynn E, Folkman J, D'Amato R. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci*. 1996;37:1625–1632.
33. Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene Therapy for Myocardial Angiogenesis : Initial Clinical Results With Direct Myocardial Injection of phVEGF165 as Sole Therapy for Myocardial Ischemia. *Circulation*. 1998;98:2800–2804.