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 Dil 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).

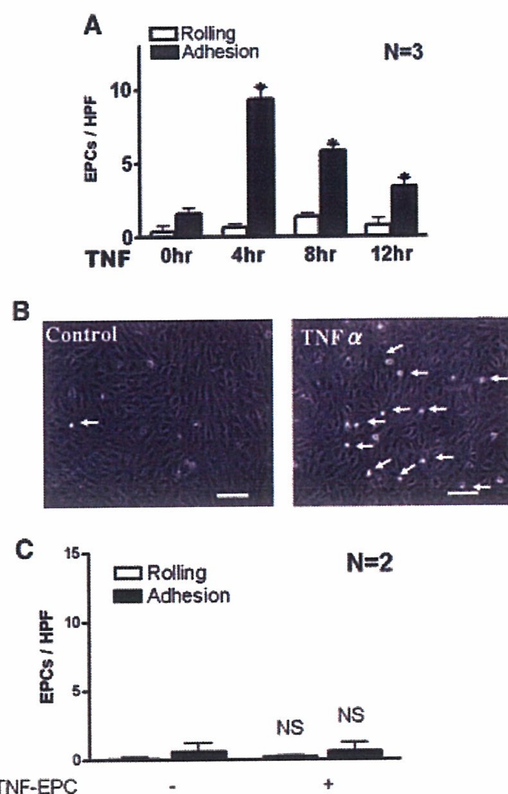

Figure 1. Adhesive interactions of EPCs on activated HUVEC monolayers under flow conditions. A, EPCs at 10 000/mL (after 7 days in culture) were perfused over HUVEC monolayers activated with tumor necrosis factor-α (5 ng/mL) for the indicated times, as described in Methods. The average number of rolling (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.

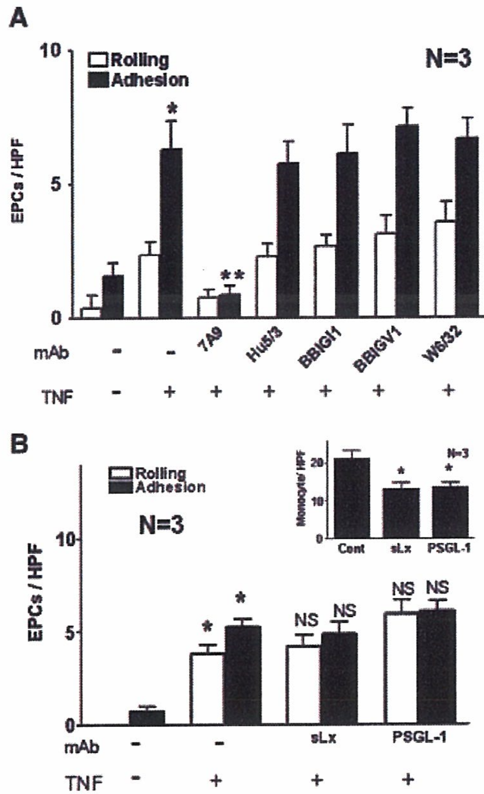

Figure 2. A, Characterization of adhesion of EPCs to HUVEC monolayers under flow conditions. Activated HUVEC monolayers (tumor necrosis factor-α, 5 ng/mL, 4 hours) were pre-incubated with mAbs against E-selectin (7A9), ICAM-1 (Hu5/3, BBIGI1), VCAM-1 (BBIGV1), and the control (W6/32, HLA-A,B,C) at 10 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

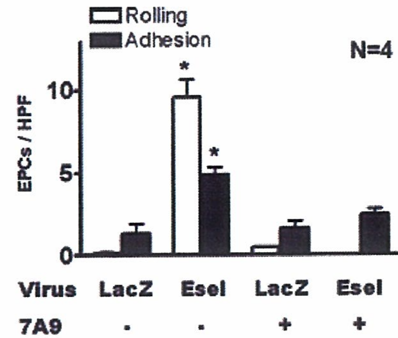

Figure 3. EPC adhesion assay using nonactivated HUVEC monolayers transduced with an E-selectin adenovirus (AdRSVE-sel) or control LacZ adenovirus (AdRSVLacZ). Each monolayer (1×10^5 cells per cover slip) was transduced with AdRSVE-sel or AdRSVLacZ at an MOI of 100 and an adhesion assay was performed 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 (≈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

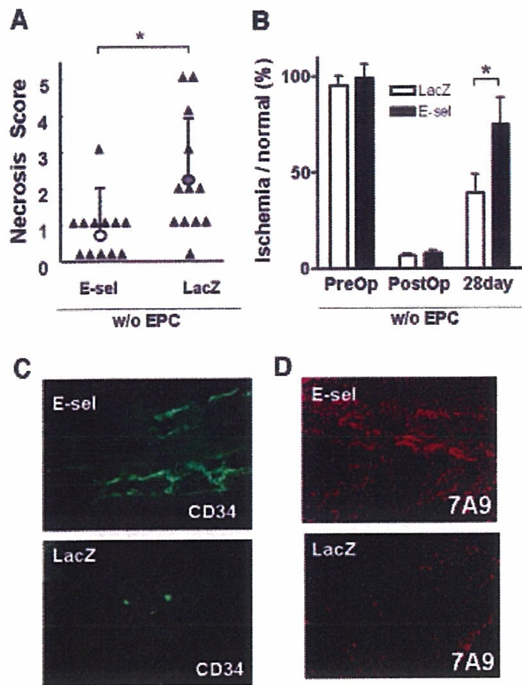

Figure 4. A, Evaluation of limb salvage on day 28, as described in Methods. Shown are the necrosis scores of mice transduced with AdRSVE-sel (E-sel, number of animals examined=12) or AdRSVLacZ (LacZ, number of animals examined=12). * $P<0.03$. B, The average rate of perfusion in each limb (ischemic/nonischemic) 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.

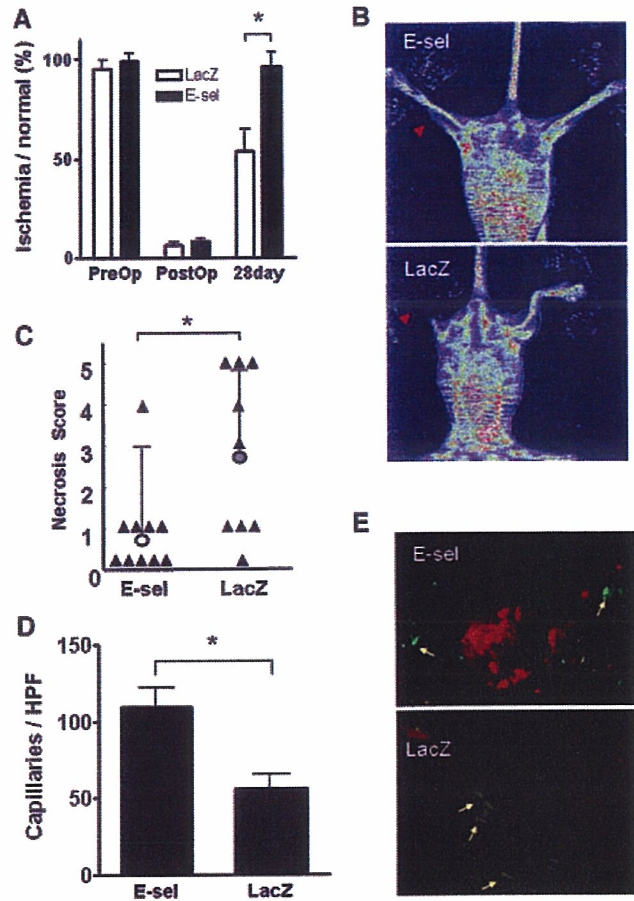

Figure 5. Contribution of human EPCs to neo-angiogenesis in hind limbs of ischemic mice. A, The average ratio of perfusion in each limb (ischemic/nonischemic) was calculated based on laser Doppler perfusion imaging analysis immediately before (PreOp) and after (PstOp) ischemic procedure and 28days after 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.

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