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Role of L-Selectin in the Vascular Homing of Peripheral Blood-Derived Endothelial Progenitor Cells

Luigi Biancone,* Vincenzo Cantaluppi,* Debora Duò,* Maria Chiara Deregibus,* Carlo Torre,† and Giovanni Camussi2*

Ex vivo expanded endothelial progenitor cells (EPCs) represent a new potential approach for the revascularization of ischemic sites. However, local accumulation of infused EPCs in these sites is poor, and the mechanisms responsible for their homing are largely unknown. We observed the expression of L-selectin, an adhesion receptor that regulates lymphocyte homing and leukocyte rolling and migration, on ex vivo expanded blood-derived human EPCs. When EPCs were subcloned in SV40-T large Ag-transfected isolates, the copresence of L-selectin and endothelial lineage markers was confirmed. We therefore demonstrated that the expression of L-selectin by EPCs was functional because it mediates interaction with a murine endothelial cell line (H.end) expressing L-selectin ligands by way of transfection with α(1,3/4)-fucosyltransferase. Indeed, adhesion of EPCs after incubation at 4°C on a rotating platform was enhanced on α(1,3/4)-fucosyltransferase-transfected H.end cells compared with control vector-transfected cells, and treatment with anti-L-selectin Abs prevented this event. We then studied the role of L-selectin in EPC homing in vivo. H.end cells were implanted s.c. in SCID mice to form endothelioma tumors, and EPCs were subsequently i.v. injected. L-selectin+ EPCs localized into α(1,3/4)-fucosyltransferase-transfected endothelial tumors to a greater extent than in control tumors, and they were able to directly contribute to tumor vascularization by forming L-selectin+ EPC-containing vessels. In conclusion, our results showed that a mechanism typical of leukocyte adhesion is involved in the vascular homing of EPCs within sites of selectin ligand expression. This observation may provide knowledge about the substrate to design strategies to improve EPC localization in damaged tissues. The Journal of Immunology, 2004, 173: 5268–5274.

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#N#oevervasculization of ischemic areas has been achieved through infusion of ex vivo purified and expanded endothelial progenitor cells (EPCs) derived from bone marrow or the circulation and exhibiting phenotypic features of endothelial cells (1, 2). Although this approach displays several attractive possibilities, its efficiency is limited by the fact that only a small fraction of the i.v. injected EPCs accumulate within sites of tissue damage, as recently demonstrated by tracking radiolabeled EPCs in mice with experimental myocardial ischemia (3). Although information has been obtained on EPC mobilization mechanisms from the bone marrow, little is known about the process that allows EPCs to insert themselves within the sites of repair (4). This phenomenon requires specific interactions among circulating EPCs and sites of vascular injury that most likely depend on the expression of homing receptors by EPCs. The investigation of homing receptors for stem and progenitor cells is of paramount importance, because it may help in the design of interventional strategies to enhance these mechanisms. In leukocyte biology, this role is played by adhesion receptors that promote their rolling on the endothelial cell surface, thus facilitating subsequent arrest and extravasation. In particular, the initial events of vascular adhesion require interaction of the selectin family of adhesion receptors with appropriately presented endothelial oligosaccharides as ligands (5). Recently, a leukocyte-restricted member of this family, L-selectin, has been found to be expressed by muscle-derived stem cells and to direct their homing into dystrophic muscles (6). Its function on leukocytes promotes their rolling on specialized endothelial cells, termed high endothelial venules (HEV), lining the postcapillary venules of peripheral lymph nodes (7, 8), which physiologically express sialylated, fucosylated, and/or sulfated L-selectin counter-receptors (9, 10). However, similar HEV structures can be observed at sites of chronic inflammation (11), where L-selectin also appears to play an important role in mediating leukocyte recruitment or on HUVEC after stimulation with TNF-α (12). In addition, the role of fucosylated oligosaccharides has been outlined by studying renal ischemia-reperfusion injury in fucosyltransferase-deficient mice in which neutrophil infiltration and tubular injury were attenuated as a consequence of the reduction in local selectin ligand endothelial expression (13). Lastly, selectin ligands have been widely detected within tumor lesions, where they may alter metastasis dissemination (14) or favor lymphocyte infiltration and antitumor immunity (15).

Based on the above data, we investigated the expression of selectins on cultured EPCs and detected L-selectin by different methods. We then proceeded to functional studies aimed at evaluating the ability of EPC L-selectin to interact with L-selectin ligand-expressing cells by using α(1,3/4)-fucosyltransferase-transfected endothelial cell lines as an adhesion substrate. Finally, we evaluated in vivo the relevance of L-selectin expression in the homing...
process by i.v. injecting EPCs in SCID mice in which α(1,3/4)-fucosyltransferase-transfected endothelial cell lines as well as control vector-transfected counterparts were s.c. injected and allowed to grow as an endothelioma tumor, as previously described (15). Accumulation of EPCs within the tumor lesions was studied by ex vivo expanded EPCs significantly contributes to their localization into tissue appropriately expressing its ligands.

Materials and Methods

Antibodies

FITC-conjugated mAb anti-human E-selectin was obtained from DakoCyto- 
tomation (Copenhagen, Denmark); FITC-conjugated mAbs anti-human CD14 (clone TUK4) and L-selectin (clone DREG-56) were purchased from Caltag Laboratories (Burlingame, CA). Purified and PE-conjugated anti-L-selectin blocking mAb (clone DREG-56) and anti-P-selectin glyco- 
protein ligand-1 (anti-PSGL-1) blocking mAb (clone KPL-1) were obtained from BD Pharmingen (San Diego, CA). Anti-human CD34 mAb was purchased from BD Biosciences (Bedford, MA), anti-human CD133 mAb was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany) and anti-human CD62P mAb was obtained from Cymbus Biotechnology (Chandler's Ford, U.K.). Isotype-matched FITC- or PE-conjugated control mouse IgG and nonimmune rabbit IgG were purchased from DakoCyto- 
tomation.

Polyclonal Abs against human von Willebrand factor (vWF), smooth muscle myosin, vascular endothelial growth factor (VEGF) recep- 
tor (VEGFR-1), VEGFR-2, VEGFR-3, Flt-3/Flik-2, Tie-2, CD62L, and 
CD62P were used for Western blot analysis or immunohistochemistry, were ob- 
tained from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-Ulex eu- 
ropaeus agglutinin-1 (UEA) and Hoechst 33258 dye were purchased from Sigma-Aldrich. FITC-mouse anti-human HLA class I Ag 1281 was obtained from Sigma-Aldrich.

Cell lines and transfectants

EPCs were isolated from PBMC from healthy donors by density centrifug- 
ation. Cells were plated on fibronectin-coated culture flasks in endothel- 
al cell basal medium-2 (Clonetics, BioWhittaker, Walkersville, MD) sup- 
plemented with endothelial growth medium (EGM-2 MV) (Cambrex, 
Walkersville, MD) single aliquots consisting of 5% FBS, VEGF, FGF-2, 
EGF, and insulin-like growth factor I. For this study EPCs from 10–30 
passes were used. Endothelial identity was studied by FACS, Western 
blot, gene microarray analysis, and functional evaluation of angiogenic 
properties. For cytofluorometric analysis, cells were detached from plates 
with EDTA, washed, resuspended in PBS, and incubated at 4°C for 30 min 
with RMI 1640 containing specific Abs. Cells were analyzed on a FACS 
(BD Biosciences, Mountain View, CA).

EPC transfectants were generated by electroporation with pBR322 plas- 
mid vector containing SV40-T large Ag gene (gift from Dr. H. L. Özer, New Jersey Medical School, Newark, NJ) (16) at 250 V and 960 μF in 
4-mm electroporation cuvettes in an electroporator II (Invitrogen Life 
Technologies, Carlsbad, CA). Clones were selected for 1 mg/ml G418 
resistance in DMEM/10% FCS.

For in vitro and in vivo adhesion experiments, murine H. end cells, previ- 
ously transfected with α(1,3/4)-fucosyltransferase IV cDNA (17) or con- 
trol vector and characterized for L-selectin binding (15), were used.

Western blot analysis

EPCs were incubated for 4 h at a lysis buffer (50 mM Tris-HCl, pH 8.3, 
containing 1% Triton X-100, 10 μM PMSF, 10 μM/ml leupeptin, and 100 
U/ml aprotinin). After centrifugation of the lysates at 15,000 × g, the 
supernatants were quantified for protein content by the Bradford method. 
Aliquots containing 100 μg of protein/lane were subjected to SDS/10% 
PAGE under reducing conditions and electroblotted onto nitrocellulose 
membrane filters. Protein array was performed by using a miniPROTEAN 
II multiscreen apparatus (Bio-Rad, Hercules, CA), which allows screening in 
single Western blots for several Abs at the same time. The blots were 
blocked with 5% nonfat milk in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 
plus 0.1% Tween (TBS-T). The membranes were subsequently 
incubated overnight at 4°C with polyclonal rabbit Ab at a concentration of 500 ng/ml. 
In selected experiments, polyclonal rabbit Ab were preincubated for 1 h at 
37°C with 20 μg/ml of the corresponding antigenic peptide provided by 
Santa Cruz Biotechnology. After extensive washing with TBS-T, the blots 
were incubated for 1 h at room temperature with peroxidase-conjugated 
protein A (200 ng/ml; Amersham Biosciences, Little Chalfont, U.K.), 
washed with TBS-T, developed with ECL detection reagents (Amersham 
Biosciences) for 1 min, and exposed to X-OMAT film (Eastman Kodak, 
Rochester, NY).

Angiogenesis assays

In vitro formation of vessel-like tubular structures was studied on growth 
factor-reduced Matrigel (8.13 mg/ml; BD Biosciences, Bedford, MA) di- 
uted 1/1 in ice-cold DMEM under a Diaphot inverted microscope (Nikon, 
Melville, NY) in a Pegasus Nikon NF-2 incubator at 37°C. Briefly, 5 × 
10^5 well cells were seeded onto Matrigel-coated wells in 10% FCS 
DMEM. After cells had attached, the FCS-containing medium was re- 
moved, and 0.5 ml of serum-free DMEM was added. Image analysis was 
performed by digital saving of images at 30-min intervals with a Micro-

Image analysis system (Casti Imaging, Venice, Italy). In vivo, angiogenesis 
was assayed as blood vessel formation from 0.5 × 10^6 EPCs embedded 
i into a solid gel of basement membrane (18). As a standard procedure, EPCs 
were resuspended into 0.5 ml of Matrigel in liquid form at 4°C, and 
then injected into the dorsal s.c. tissue of 8-wk-old SCID mice (n = 3). 
Three days later, mice were killed, and Matrigel plugs were processed for light 
microscopy.

Gene array

Human GEArray kits for the study of angiogenesis and adhesion molecules 
(SuperArray, Bethesda, MD) were used to characterize the gene expression 
profiles of EPCs and normal HUVECs. Hybridization was performed ac- 
cording to the manufacturer’s instructions, as previously described (19). 
Briefly, total RNA was extracted using the TRI-Reagent (Sigma-Aldrich), 
from EPCs or HUVECs cultured at subconfluence in the absence of growth 
factors. RNA pooled from different cultures was used as a template for 
biotinylated probe synthesis. For probe synthesis, each RNA sample (5–10 
μg) was combined with a primer mix and with 100 U of reverse transcrip-
tase (200 U/μl; Promega, Madison, WI), 8 U of RNase inhibitor (4 U/μl; 
Promega) and a dNTP mix with biotin-16-dUTP (12 μM; Roche, Indi-

Biosciences) for 1 min, and exposed to X-OMAT film (Eastman Kodak, 
Rochester, NY) for 30 min. After the induction period, nonadherent EPCs were removed by washing three 
times with the incubation medium. In some experiments, EDTA to 10 mM 
was added to the plate (at 2 

°C for 30 min. After the 
incubation periods, nonadherent EPCs were removed by washing three 
times with PBS, counted on a hemocytometer, and resuspended to 10^6 
/ml for evaluation of the binding of L-selectin to PSGL-1, HL60 cells 
(American Type Culture Collection, Manassas, VA) were labeled over- 
night with 60 μM 3',3'-diodecylxyllocarbocyanine (Molecular Probes, 
Eugene, OR) in RMI 1640 supplemented with 5% FCS, washed three 
times with PBS, counted on a hemocytometer, and resuspended to 10^7/ml

Adhesion assay

Adhesion of EPCs on H. end transfectants was studied in nonstatic condi-
tions according to the method described by Sperin et al. (20). Cells were 
labeled overnight with the green fluorescent cell linker PKH2 (Sigma-
Aldrich), which maintains the fluorescence staining for >100 mitotic di-
visions. After centrifugation at 1400 × g for 10 min, cells were resus- 
pended in Tri-buffered Tyrode’s solution containing 0.5% human serum 
albumin. H. end transfectants, grown to confluence in 24-well plates, were 
wash three times with RMI 1640 medium containing 0.5% human serum 
albumin and placed on a platform rotator (80 rpm), and EPCs were 
added to the plate (at 2 × 10^3 cells/well) at 4°C for 30 min. After the 
incubation period, nonadherent EPCs were removed by washing three 
times with the incubation medium. In some experiments, EDTA to 10 nM 
was added to the wells or EPCs were preincubated for 10 min at 4°C with 
20 μg/ml anti-L-selectin blocking mAb (clone DREG-56) before the co- 
incubation. In parallel experiments, a wound was introduced by means of 
a cell scraper within H. end transfectant monolayers. After incubation for 
30 min, nonadherent EPCs were removed by washing three times with 
the incubation medium. For evaluation of the binding of L-selectin to PSGL-1, HL60 cells

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in Iscove’s medium containing 0.25% BSA. Cells were then added to confluent monolayers of EPCs plated in 60-mm diameter plastic dishes. Experiments were conducted at 4°C for 30 min. After the incubation periods, nonadherent HL60 cells were removed by washing three times with the incubation medium. In selected experiments, 10 μg/ml anti-PSGL-1 mAb or an irrelevant control IgG were added to the HL60 cells 30 min before incubation with EPCs. Samples were then fixed with 3% formaldehyde/PBS and observed under an epifluorescence microscope. Bound fluorescent green HL60 cells were counted. Experiments were performed in triplicate, and 10 fields at ×400 magnification per sample were evaluated.

**Evaluation of EPC homing in vivo**

End transfectants (0.5 × 10^7 cells) in a total volume of 150 μl were injected s.c. into the left back of SCID mice via a 26-gauge needle, using a 1-ml syringe to form tumors similar to hemangiosarcomas, as previously described (15). Three days later, 10^7 EPCs were injected i.v. through the tail vein. Before injection, cells were labeled with the green fluorescent cell linker PKH2 (Sigma-Aldrich) for tail vein. Before injection, cells were labeled with the green fluorescent cell linker PKH2 (Sigma-Aldrich). Fluorescent cells per field (magnification, ×200) were counted in 10 fields per specimen, and the mean ± SD were calculated.

**Tissue preparation for scanning electron microscopy**

Samples were fixed in 2.5% paraformaldehyde containing 2% sucrose, and cell cultures were processed as previously described (21). Tissue samples were snap-frozen to hatching in liquid nitrogen. Immunogold labeling was performed as described previously (22) using a specific primary Ab (clone DREG-56 anti-human L-selectin mAb) and, as secondary Ab, 5 nm gold-conjugated goat anti-mouse (BB International, Cardiff, U.K.), followed by silver enhancement (silver enhancing kit; BB International). As a control, primary Ab was replaced by an isotype-matched Ig. Samples were then postfixed in 2.5% glutaraldehyde, dehydrated in alcohol, dried, and coated with carbon or gold by sputter coating. The specimens were examined in a scanning electron microscope (LEO 1430 VP; LEO-Electron Microscope, Cambridge, U.K.). Images were obtained via secondary electron or backscattered electron detection at a working distance of 15–25 nm and an accelerating voltage of 14–26 kV.

**Confocal fluorescence microscopy**

For double-staining colocalization studies, EPCs were plated into a glass chamber slide system (Lab-Tek, Naperville, IL) and grown to 40% confluence. Then cells were washed with PBS, fixed for 10 min with 8% paraformaldehyde, and permeabilized for 10 min with a 0.1% Triton X-100/PBS solution. After 1-h incubation with PBS containing 1% BSA, cells were incubated for 1 h at room temperature with anti-vWF Abs or rabbit IgG, followed by FITC-conjugated secondary Abs for 30 min or FITC-conjugated UEA-1 lectin. Slides were then extensively washed with PBS and incubated with PE-conjugated anti-L-selectin mAb for 1 h or with an irrelevant PE-conjugated mouse IgG. At the end of the incubation, cells were washed, and Hoechst 33258 dye was added for nuclear staining. Slides were mounted with a glycerol/PBS solution and observed under a Leica TCS SP2 model confocal microscope (Heidelberg, Germany).

**Results**

Flow cytometric analysis revealed L-selectin on CD14⁺ blood-derived EPC cultures (Fig. 1A). Expression of the other selectin family members, CD62E and CD62P, was negative (number of positive cells, <1%). Consistent with several reports (23, 24), cultured EPCs were negative for CD34 and CD133 expression (not shown). Peripheral blood leukocytes from healthy volunteers and the human microvascular endothelial cell line HMEC were used for L-selectin-positive and negative controls, respectively (not shown). L-selectin expression was confirmed by Western blot analysis of EPC lysates (Fig. 1C), and L-selectin mRNA was detected within an adhesion molecule gene microarray panel analysis of EPCs (Fig. 1D). In addition, immunostain for L-selectin colocalized with UEA-1 binding (Fig. 1E) that detects α-L-fucosyl residues on surface glycoproteins expressed on endothelial cells and recently demonstrated on EPCs (24) and with vWF. Lastly, immune-scanning electron microscope confirmed L-selectin expression and showed diffuse distribution on the cell surface (Fig. 1, F–I).

**FIGURE 1.** Expression of L-selectin on cultured EPCs. A and B, Cytosfluorometric studies showed the expression of L-selectin (A, continuous line), but not of CD14 (B, continuous line), vs isotopic irrelevant control (dotted line). C, L-selectin expression was confirmed by Western blot analysis of lysates from cultured EPCs (lane 1, EPCs; lane 2, m.w. markers). D, A gene microarray assay for human extracellular matrix and adhesion molecules detected L-selectin-specific mRNA (row k, lane 1), PUC18 plasmid DNA served as negative controls in row m, lanes 1–3, whereas GAPDH, cyclophilin A, ribosomal protein L13a, and β-actin were used as positive controls in row m, lanes 7 and 8; row n, lanes 1–4; row n, lanes 5 and 6; and row n, lanes 7 and 8, respectively. Complete gene list and position information are available at www.superarray.com. E, Immunostaining of EPCs with PE-conjugated anti-L-selectin colocalized with FITC-conjugated UEA-1 binding that detects α-L-fucosyl residues on surface glycoproteins expressed on endothelial cells and on EPCs (E; magnification, ×400) and with vWF (magnification, ×400). Nuclei were counterstained in blue with Hoechst 33258 dye. F–I, Immunogold scanning electron microscopy of EPCs stained with anti-L-selectin mAb (F, secondary electron detection; G, backscattered electron detection; H, secondary electron detection; J, backscattered electron detection; magnification, ×750) or with a control isotype-matched Ab (H, secondary electron detection; J, backscattered electron detection; magnification, ×750). In G, the backscattered detection shows the granular bright deposits corresponding to gold particles, whereas the border of the cells is undistinguishable. The granular deposits are absent in the control specimen (I).
and G) compared with the control Ab (Fig. 1, H and E). The back-scattered detection that shows only the granular positivity of gold particles shows the specific binding of gold-labeled Abs to the cells (Fig. 1G) compared with the control (Fig. 1G). The endothelial phenotype was further studied by Western blot analysis that demonstrated expression of characteristic endothelial markers such as Tie-2, VEGFR-2, and VEGFR-3, but not of VEGFR-1 (Fig. 2A). We therefore evaluated whether L-selectin-expressing EPCs retained proangiogenic properties in vitro and in vivo. When plated on Matrigel-coated wells, EPCs rapidly formed tubular structures 4 h after seeding (Fig. 2B). In an in vivo model of angiogenesis, 0.5 × 10⁶ EPCs were implanted s.c. in 0.5 ml of Matrigel into SCID mice (n = 3). Histological examination of the Matrigel plugs 3 days after implantation showed extensive capillary network formation performed by EPCs (Fig. 2C). Cells present in the vessels were in large part EPCs, because they stained with a mouse anti-human HLA class I mAb (Fig. 2D).

To further dispel the possibility that L-selectin detection in EPCs cultures depended on the presence of a contaminating monocytic/macrophage population, EPCs were subcloned by transfection with pBR322 plasmid vector containing the SV40-T large Ag gene (17) and the neomycin resistance gene, and three cell isolates were raised from single colony wells after G418 selection. As shown in Fig. 3, these clonal isolates expressed L-selectin and vWF, Tie-2, VEGFR-2, VEGFR-3, and Flt-3/Flk-2, but not the macrophage marker CD14. Confocal microscopic analysis showed colocalization of UEA-1 binding and L-selectin in the same cells (Fig. 3C).

FIGURE 2. Antigenic and functional characterization of cultured EPCs. A, Protein array performed by Western blot analysis using a Mini Protean multiscreean apparatus. Cell lysate was blotted with different Abs against (lanes 1–6, respectively): irrelevant control, smooth muscle myosin, VEGFR-2, VEGFR-3, VEGFR-1, and Tie-2. B and C, In vitro and in vivo angiogenesis assays. When plated on Matrigel-coated wells, EPCs rapidly (4 h) formed tubular structures (B; magnification, ×250). In vivo, 0.5 × 10⁶ EPCs were implanted s.c. in 0.5 ml of Matrigel into SCID mice; extensive capillary network formation was observed at the histologic analysis 3 days after implantation (C; magnification, ×200). In D (magnification, ×200), staining with a human-specific anti-HLA class I Ag mAb showed that cells present in the vessels are human cells. Nuclei were counterstained with propidium iodide. Similar results were obtained in three independent experiments.

FIGURE 3. L-selectin expression and characterization of SV40-T large Ag immortalized EPCs. A–B, FACS analysis showing absence of CD14 (A) and expression of L-selectin (B) in a representative SV40-T large Ag immortalized EPC line (irrelevant Ab control as dotted line vs specific Ab staining as continuous line). C, Detection of villous adhesion to an altered endothelial layer. For functional evaluation of L-selectin expression in this setting, EPC adherence to endothelial cells was studied. Under normal conditions, endothelial cells (with the exception of HEV) do not express functional L-selectin ligands. Therefore, we used the previously developed H.endft transfectants stably expressing α(1–3/4)-fucosyltransferase, which catalyzes transglycosylation reactions, yielding both Fuc(1,3)- and Fuc(1,4)-glycosidic bonds (17), and directs expression of functional L-selectin ligands. As control, parental H.end cells transfected with the hygromycin resistance selection vector only (H.endhygro) that do not express fucosylated L-selectin ligands were used (15). Adhesion experiments were performed in specific conditions (at 4°C on a rotating platform) to study selectin-mediated cell interactions (20). EPCs were observed to adhere to H.endft, but very scarcely to control H.endhygro cells (Table I and Fig. 4). The enhanced EPC adhesion to H.endft monolayers was L-selectin- and Ca²⁺-dependent, as shown by the inhibitory effect of anti-L-selectin mAb and of EDTA, respectively (Table I). In a wounded monolayer system, where cocultures were

It is conceivable that circulating EPCs localize into sites of vascular injury in a multiple step process that most likely initiates with their interaction with an altered endothelial layer. For functional evaluation of L-selectin expression in this setting, EPC adherence to endothelial cells was studied. Under normal conditions, endothelial cells (with the exception of HEV) do not express functional L-selectin ligands. Therefore, we used the previously developed H.endft transfectants stably expressing α(1–3/4)-fucosyltransferase, which catalyzes transglycosylation reactions, yielding both Fuc(1,3)- and Fuc(1,4)-glycosidic bonds (17), and directs expression of functional L-selectin ligands. As control, parental H.end cells transfected with the hygromycin resistance selection vector only (H.endhygro) that do not express fucosylated L-selectin ligands were used (15). Adhesion experiments were performed in specific conditions (at 4°C on a rotating platform) to study selectin-mediated cell interactions (20). EPCs were observed to adhere to H.endft, but very scarcely to control H.endhygro cells (Table I and Fig. 4). The enhanced EPC adhesion to H.endft monolayers was L-selectin- and Ca²⁺-dependent, as shown by the inhibitory effect of anti-L-selectin mAb and of EDTA, respectively (Table I). In a wounded monolayer system, where cocultures were
transposed at 37°C for 4 h after incubation for 30 min at 4°C on a rotating platform, insertion of EPCs was markedly enhanced into H.endft monolayer wound compared with the H.endhygro counterpart (Fig. 5). We, therefore, evaluated the functional activity of L-selectin expressed by EPCs on the binding to PSGL-1, a major L-selectin ligand expressed on leukocytes (25). For this purpose, we incubated EPCs with HL60 cells, which are known to express PSGL-1, at 4°C on a rotating platform and evaluated their adhesion. As shown in Fig. 6, when cells were incubated with an anti-PSGL-1 mAb, a significant reduction in HL-60 cell adhesion was observed.

For evaluating in vivo the relevance of this mechanism, we s.c. implanted H.end cells in SCID mice to form tumors similar to endothelioma, as previously described (15). In this tumor type, a relevant part of the tumor vessel endothelium is constituted by H.end cells themselves (26). An equal number of cells (0.5 × 10^7) from each transfectant (H.endhygro and H.endft) was injected s.c. in SCID mice (six per group), and the animals were monitored for visible tumor growth. Both H.endhygro and H.endft cells formed tumors of similar size (~10 mm) in 3 wk. Three days before death, 10^7 EPCs were injected i.v. through the tail vein. Before injection, cells were labeled with the vital fluorescent dye PKH2 for detection. At the end of the experiment, tumor tissue was processed for fluorescence, histology, immunohistochemistry, and electron microscopy. Fluorescence examination demonstrated the presence of several EPCs within the tumor tissue derived from H.endft cells (Fig. 7A). EPCs were detected as scattered cells distributed within the tumor tissue as well as inserted into vessels. Fluorescent cell count per microscopic field demonstrated a significantly higher number of EPCs located within H.endft tumors than in H.endhygro control counterparts (Table II). Then we performed immunoscanning electron microscopy for human L-selectin to further evaluate the role of the homed L-selectin** EPCs. Immunogold-labeled EPCs were detected within patent vessels of H.endft tumors as part of the endothelial layer (Fig. 7, D and E). This aspect was not observed in all the H.endhygro tumor fragments examined (Fig. 7, F and G).

Discussion

It is well established that circulating bone marrow-derived endothelial cell progenitors may locate specifically within sites of endothelial damage and repair (27). However, the mechanisms responsible for their homing are only partially known. We analyzed the cell surface molecule repertoire of EPC cultures and detected L-selectin expression. This receptor plays a critical role in the initial events of lymphocyte trafficking from the circulation to lymphoid organs or extralymphoid tissues (9). Interaction between leukocyte L-selectin and endothelial cell ligands, which are composed of appropriately presented sialylated, fucosylated, and/or sulfated oligosaccharides (11), promotes leukocyte rolling on the endothelial surface, facilitating the subsequent leukocyte arrest and the extravasation (6). L-selectin has been described to date as only expressed by leukocytes or leukocyte-derived tumor lineages (8). However, L-selectin was recently also detected on the surface of muscle-derived stem cells (7). Most notably, L-selectin was required for the homing of stem cells into dystrophic muscles that display expression of L-selectin ligands on blood vessel endothelium. Beside lymph nodes, detection of L-selectin ligands on diseased tissues and tumors is a consolidated idea. Indeed, inflamed sites (17) as well as ischemic areas (13) show the expression of L-selectin ligands as a consequence of fucosyltransferase activation. These elements constituted a rationale for investigating L-selectin expression on EPCs in the effort to elucidate the homing mechanisms of these cells into diseased tissues.

![FIGURE 4](image-url)

**Representative scanning electron microscope micrographs showing adhesion of EPCs on H.endft (A) or H.endhygro cells (B) after incubation at 4°C for 30 min on a platform rotator (80 rpm) and extensive washings to remove nonadherent cells (A and B magnification, ×1000).**

![FIGURE 5](image-url)

**Adhesion of fluorescent EPCs to a wound introduced into H.endft (A) or H.endhygro cell (B) monolayers. Cocultures were transposed at 37°C for 4 h after incubation for 30 min at 4°C on a rotating platform and washings (A and B magnification, ×200). Three independent experiments were performed with similar results.**

### Table 1. Adhesion of EPCs on H.end transfected monolayers

<table>
<thead>
<tr>
<th>EPCs</th>
<th>H.endhygro</th>
<th>H.endft</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPCs alone</td>
<td>25.6 ± 3.78</td>
<td>57.6 ± 5.63</td>
</tr>
<tr>
<td>EPCs + 10 mM EDTA</td>
<td>28.6 ± 2.51</td>
<td>22.6 ± 4.04</td>
</tr>
<tr>
<td>EPCs + anti-L-selectin mAb (DREG-56)</td>
<td>24.0 ± 4.58</td>
<td>19.6 ± 4.72</td>
</tr>
</tbody>
</table>

*In vitro adhesion assay of EPCs to H.endhygro and H.endft. PKH2-labeled EPCs were added to H.endhygro and H.endft cell monolayers (at 2 × 10^5 cells/well). Adhesion assays were performed as described in Materials and Methods. Fluorescent cells in 10 fields per well were counted at ×200 magnification and number are representative of mean ± SD of three separate experiments performed in triplicate. ANOVA with Newman-Keuls multi-comparison test was performed. Statistical differences (p < 0.05) were encoded as follows: H.endhygro alone vs H.endft alone (*); H.endhygro alone vs H.endhygro with EDTA or anti-L-selectin mAb (not significant); H.endft alone vs H.endft with EDTA or anti-L-selectin mAb (**).
In the present study we detected L-selectin-positive cells in ex vivo expanded EPC cultures by multiple assays. Our culture conditions for total mononuclear cell fractions led to the expansion of CD14− populations that expressed Tie-2, vWF, VEGFR-2, and VEGFR-3 endothelial cell markers. These cells derive from a late outgrowth of a distinct CD14− population similar to what was recently described by Gulati et al (23). We observed little initial proliferation of adherent CD14− cells, followed by late cell expansion from small colonies that started only 3–4 wk later. We analyzed this cell population after 10 passages and confirmed its endothelial phenotype. The absence of a CD14 macrophage cell marker indicated that the detection of L-selectin in EPC preparations was not related to a contaminating macrophage population. From the functional point of view, these cells displayed angiogenic properties as they form vessel-like structures in vitro and, most importantly, are able to colonize and revascularize Matrigel plugs in vivo (28). This cell population possibly corresponds to the endothelial phenotype of circulating cells capable of assuming an endothelial phenotype and properties. Notably, within the heterogeneity in the population of circulating cells capable of assuming an endothelial phenotype, we detected L-selectin expression on probably the most suitable cell type for therapeutic revascularization.

The expression of L-selectin on EPCs was functional to mediate interaction with selectin ligand-expressing endothelial monolayers. Indeed, blocking experiments with anti-L-selectin Abs completely abrogated EPC adhesion to endothelial cells under non-static conditions at 4°C. This result together with the absence of P- and E-selectin on the EPC surface suggests that L-selectin may uniquely mediate interaction with fucosylated selectin ligands expressed by endothelial cells. In addition, the physical EPC-endothelial interaction mediated by L-selectin enabled EPC insertion within the monolayer when the cultures were transposed at 37°C.

In vivo, we observed recruitment of EPCs within endotheliomas expressing appropriate fucosylation. In contrast, basal EPCs accumulated in control H.end endotheliomas was poor. In this experimental group EPCs were never found inserted in vessel endothelium, but mostly as isolated cells, conceivably arrested by plugging small caliber capillaries. The lack of several receptor-ligand interactions in a xenogeneic setting and the short time of exposure to circulating EPCs may account for the low level of basal engraftment of EPC in this model compared with that observed in other studies (30, 31). However, this was probably instrumental to better dissect the effect of selectin-mediated interactions that are cross-species.

Compared with leukocytes, L-selectin was expressed at a lower level on EPCs. It is therefore possible that enforcement of L-selectin expression by gene transfer during ex vivo expansion may increase their homing ability within sites of injury. Furthermore, this approach may be used, in general, to increase the accumulation of stem and progenitor cells in diseased tissues where the expression of selectin ligands occurs.

In conclusion, our study indicates that ex vivo-expanded EPCs express L-selectin and that this receptor may direct recruitment of EPCs to specific anatomical sites that present appropriate ligands.

**Table II. Analysis of tumor-infiltrating EPCs**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fluorescent cells/field*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.end/hygro tumors</td>
<td>4.2 ± 2.06*</td>
</tr>
<tr>
<td>H.end tumors</td>
<td>16.6 ± 6.25</td>
</tr>
</tbody>
</table>

* H.end/hygro tumors and H.end tumors were examined 3 days after i.v. inoculation of 10^7 PKH2-labeled EPCs in SCID mice (six mice per group). Ten fields were counted at magnification ×200 and numbers are representative of mean ± SD of tumor-infiltrating EPCs counted in six mice per group. Statistical analysis was performed for H.end/hygro tumors vs H.end tumors (Student’s t test).

*<i>p < 0.01.</i>
This may occur at the sites of inflammation/immune injury, ischemia, and tumor angiogenesis.

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References


