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Endothelial progenitor cell dysfunction in diabetes mellitus

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Chapter 2

CD34⁺ Cells Home, Proliferate and Participate in Capillary Formation, and in Combination With CD34⁻ cells Enhance Tube Formation in a 3-Dimensional Matrix

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Abstract

Objective

Emerging evidence suggests that human blood contains bone marrow (BM)-derived endothelial progenitor cells that contribute to postnatal neovascularization. Clinical trials demonstrated that administration of BM-cells can enhance neovascularization. Most studies, however, used crude cell populations. Identifying the role of different cell populations is important for developing improved cellular therapies.

Methods and Results

Effects of the hematopoietic stem cell-containing CD34⁺ cell population on migration, proliferation, differentiation, stimulation of, and participation in capillary-like tubule formation were assessed in an *in vitro* 3-dimensional matrix model using human microvascular endothelial cells. During movement over the endothelial monolayer, CD34⁺ cells remained stuck at sites of capillary tube formation and time- and dose-dependently formed cell clusters. Immunohistochemistry confirmed homing and proliferation of CD34⁺ cells in and around capillary sprouts. CD34⁺ cells were transduced with the LNGFR marker gene to allow tracing. LNGFR gene-transduced CD34⁺ cells integrated in the tubular structures and stained positive for CD31 and UEA-1. CD34⁺ cells alone stimulated neovascularization by 17%. Coculture with CD34⁻ cells led to 68% enhancement of neovascularization, whereas CD34⁻ cells displayed a variable response by themselves. Cell-cell contact between CD34⁺ and CD34⁻ cells facilitated endothelial differentiation of CD34⁺ cells.

Conclusions

Our data suggest that administration of CD34⁺-enriched cell populations may significantly improve neovascularization and point at an important supportive role for (endogenous or exogenous) CD34⁻ cells.

Introduction

The formation of new capillaries plays a critical role in physiological and pathological processes such as wound healing, ischemia and tumor growth. It has long been thought that postnatal neovascularization occurred exclusively by migration and proliferation of preexisting endothelial cells (angiogenesis). Increasing evidence indicates that bone marrow (BM)-derived circulating endothelial progenitor cells (EPCs) are also involved in postnatal new vessel formation a process that¹, reminiscent of embryonic vessel formation, is termed adult vasculogenesis². The concept of 'therapeutic vasculogenesis', administration of adult progenitor cells or progenitor-containing cell populations to stimulate neovascularization, and the potential of progenitor cells to serve as new vehicles for gene therapy has received a lot of scientific attention. Several small clinical trials aimed at therapeutic vasculogenesis by autologous transplantation of BM cells have been performed and improved clinical outcomes in patients with severe chronic limb ischemia or myocardial ischemia have been reported³⁻⁶.

An important question concerning therapeutic vasculogenesis is, which cell population should be administered? Thus far, most clinical studies have used nonselected BM mononuclear cells; however, administration of such crude cell populations may have unwanted side effects. Recent data suggest that beside EPCs, BM contains other progenitor cells that may contribute to atherosclerosis⁷, whereas hematopoietic cells were reported to have the capacity to produce profibrotic and angiogenic factors^{8,9}. Better characterization of the BM cell subpopulation that can generate EPCs is of critical importance to development of safer and better-targeted cellular therapies.

Several studies have suggested that in human postnatal life, analogous to the existence of the embryonic hemangioblast¹⁰, the CD34⁺ hematopoietic stem cell population contains cells that can give rise to EPCs and endothelial cells^{1,11,12}. This would suggest the potential use of CD34⁺-enriched cell populations in therapeutic vasculogenesis. Various authors reported that administration of human leukocytes enriched for CD34⁺ cells effectively enhanced neovascularization in animal models^{1,13,14}. However, it has been argued that, because of lack of purity of the CD34⁺-enriched cell populations and use of vital dye labeling of CD34⁺ cells with possible dye transfer among cells, definitive proof that CD34⁺ cells are angioblasts is lacking¹⁵. Several investigators pointed at a more important role for the CD34⁺CD14⁺ monocytic cell population¹⁵⁻¹⁹. Interestingly, recent reports suggest that

the majority of blood-derived cultured acetylated low-density lipoprotein (LDL) and *Ulex europaeus* double-positive cells, which are commonly referred to as EPCs, may be derived from monocytes/macrophages²⁰. We previously demonstrated that a minor but significant subset ($\approx 9\%$) of cultured EPCs originates from the CD34⁺ mononuclear cell population²¹. It has been suggested that EPCs derived from the monocyte/macrophage cell population exert their beneficial proangiogenic effects mainly by growth factor secretion and should be more appropriately called circulating angiogenic cells (CACs), whereas the hematopoietic stem cell-related populations may yield 'late outgrowth EPCs' in culture, also referred to as 'the true EPCs,' enhancing neovasculogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency²⁰⁻²².

The present study was conducted to determine the exact role of CD34⁺ cells in human neovascularization. Thus far, most studies on human vasculogenesis were performed with human cells in small animal models lacking an intact immune system. We have used a 3-dimensional (3D) *in vitro* neovascularization model to study the role of human CD34⁺ cells in vasculogenesis and angiogenesis in a human microvascular endothelial system under controlled circumstances. This model provides a tool to dissect the different mechanisms involved: migration, proliferation, differentiation, stimulation of, and participation in capillary formation.

Methods

Materials

Histopaque 1077, diaminobenzidine (DAB), KDR antibodies (Clone KDR1) and tetramethyl rhodamine isothiocyanate (TRITC)-labeled *Ulex europaeus* were obtained from Sigma Chemicals, St. Louis, MO; DiI-labeled acLDL from Biomedical Technologies Inc, Stoughton, MA; histofine AEC solution from Nichirei, Tsukiji, Chuo-Kutokyo, Japan; VE-cadherin antibodies (clone BV6:MAB1989) from Chemicon, Temecula, CA; eNOS antibodies from Transduction Laboratories, Lexington, KY; fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (clone8G12) antibodies, phycoerythrin (PE)-labeled goat anti-mouse IgG1-antibodies, FITC-labeled polyclonal goat anti-mouse antibodies and the Calibur flow cytometer from Becton Dickinson, Mountain View, CA; mouse anti-human CD31 antibodies, and anti-Ki67 antibodies (clone MIB-1) from DAKO, Glostrup,

Denmark; powervision goat anti-mouse poly-HRP-conjugates from Immunovision Technologies, Springdale, AR; FITC-conjugated streptavidin, TRITC-conjugated streptavidin, biotin blocking kit, ABC-mouse-kit, normal horse serum, biotinylated anti-mouse IgG, avidin DH solution, biotinylated peroxidase and vectashield from Vector Laboratories, Burlingame, CA; the magnetic cell sorter (MACS) from Miltenyi Biotec, Gladbach, Germany; StemPro-34-SFM medium (SCM), heat-inactivated newborn calf serum (NBCS) and fetal calf serum (FCS) from GibcoBRL, Grand Island, NY; stem cell factor (SCF), flt-3 ligand (FL), thrombopoietin (TPO) and basic fibroblast growth factor (bFGF) from Pepro Tech, Rocky Hill, NY; mouse anti-human LNGFR antibodies (clone 20.4) and the lymphoblast cell line Raji from ATCC, Manassas, VA; penicillin, streptomycin and medium 199 (M199) from Biowittaker, Verviers, Belgium; L-glutamine from ICN, Costa Mesa, CA; heparin from Leo Pharmaceutic Products, Weesp, The Netherlands; 0.4µm pore trans-well system from Costar, Cambridge, MA. Tumor necrosis factor- α (TNF- α) was a kind gift of Dr J. Tavernier, Biogent, Gent, Belgium. Blocking antibodies against P-selectin (AF137), E-selectin (AF724), VCAM (BBA5) and ICAM-1 (BBA3) were all obtained from R&D Systems, Minneapolis MI.

Isolation of human CD34⁺ cells

Mononuclear cells (MNCs) were isolated from human umbilical cord blood (hCB) by density centrifugation (Histopaque 1077). Cells were washed and CD34⁺ cells were isolated by magnetic bead separation method (MACS). The residual cells, depleted from CD34⁺ cells, served as CD34⁻ cell population. The purity of the cell populations was analyzed by flow cytometry. Protocols for sampling hCB were approved by the institutional ethical committee. Written informed consent was obtained from all mothers before labor and delivery.

Genetic marking of CD34⁺ cells

CD34⁺ cells were cultured for 2 days with StemPro-34-SFM medium containing stem cell factor, flt3 ligand and thrombopoietin. Genetic marking was performed as described previously²³ using a retrovirus encoding the truncated low-affinity nerve growth factor receptor (LNGFR) as a marker gene. After transduction, CD34⁺ cell selection was repeated as described to remove cells that had lost CD34 expression during transduction. Transduction efficiency and CD34⁺ selection were evaluated by flow cytometry.

Flow cytometry

After MACS separation of CD34⁺ cells the different cell populations were analysed by FACS. To evaluate the purity of the sorted cell populations, 25000 cells were stained FITC-conjugated anti-human CD34 and analyzed using a Calibur flow cytometer. As negative control the first antibody was substituted with an irrelevant murine IgG of the same subclass.

After transfection of CD34⁺ cells with LNGFR, CD34⁺ cell selection was repeated to remove cells that had lost CD34 expression during transduction. Aliquots of 25000 cells were incubated with mouse anti-human LNGFR-antibody. A PE-labeled goat anti-mouse IgG1-antibody was used as secondary antibody. Subsequently cells were incubated with FITC-conjugated anti-human CD34-antibody. Controls were included by substituting the primary antibody by an irrelevant antibody of the same subclass.

Differentiation and characterization of EPC/CAC

EPC/CAC were obtained as previously described²⁴ by culturing hCB-MNC at a density of 2×10^6 cells/cm² on a gelatin-coated 6-well plate in M199 medium containing 20% fetal calf serum, penicillin/streptomycin, endothelial cell growth factor (ECGF), and heparin. At day 7, nonadherent cells were removed by thorough washing. Adherent cells were harvested and used in the 3D neovascularization assay as described.

To confirm endothelial phenotype, a subset of EPC/CAC were cultured on gelatin-coated coverslips for 7 days, fixed in cold methanol (-20°C) and immunostained for the expression of the endothelial markers vascular endothelial (VE)-cadherin, endothelial nitric oxide synthase (eNOS) and kinase insert domain receptor (KDR) was performed using the ABC-mouse-kit. After blocking with normal horse serum for 30 minutes cells were incubated with the primary antibody (VE-cadherin, eNOS, KDR) at 37°C for 1 hour, followed by incubation with biotinylated anti-mouse IgG and normal horse serum for 1 hour at 37.5°C. Subsequently, the cells were incubated in 2% avidin DH solution and 2% biotinylated peroxidase for 1 hour at 37°C. As chromogen the histofine AEC solution was used. To assess the ability to take up acetylated LDL (acLDL) cells were incubated with DiI-labeled acLDL for 4 hours at 37°C and after fixation were incubated with FITC-labeled *Ulex europaeus* agglutinin-I for 1 hour. For CD31-staining the fixed cells were incubated with mouse anti-human CD31 antibody for 1 hour, followed by incubation with FITC-labeled polyclonal goat anti-mouse antibodies. For all stainings controls were included in which the primary antibody was replaced by PBS or an irrelevant antibody of the same subclass.

In vitro neovascularization assay

Human foreskin MVEC were isolated, cultured, and characterized as described previously²⁵. Cells were cultured until confluence at 5% CO₂/95% air on gelatin-coated dishes in M199 medium, supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum (serum pooled from 10 donors, obtained from a local blood bank), 10% heat-inactivated newborn calf serum (NBCS), 150 µg/mL endothelial cell growth factor (ECGF, prepared from bovine brain), 5 IU/mL heparin, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C. Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3. The experiments were performed with confluent cells (0.7*10⁵ cells/cm²) from passage 10-11 that were cultured without growth factor for at least 24 hours.

The *in vitro* neovascularization assays were performed in human fibrin matrices as described previously²⁶. In short, highly confluent hMVECs (0.7*10⁵ cells/cm²) were seeded in a 1.25:1 split ratio on fibrin matrices and cultured in M199 medium supplemented with 10% human serum, 10% newborn calf serum, penicillin/streptomycin, basic fibroblast growth factor (bFGF) and tumor necrosis factor- α (TNF- α). After 24 hours, the medium was replaced with medium containing the mediators, with or without different cell populations. Fresh medium was added every second day. Invading cells and tubular structures of hMVECs in the 3D fibrin matrix were analyzed by phase-contrast microscopy. The length and amount of the tube-like structures was determined using an Olympus-CK2 microscope equipped with a monochrome charge-coupled device camera (MX5) connected to a computer with Optimas image analysis software. Six fixed microscopic fields (7.3 mm² per field) per well were analyzed and used to calculate the total length of the tube-like structures, expressed as mm/cm².

The effects of CD34⁺ cells on in vitro capillary formation: homing to foci of neovascularization

To compare different cell populations for their migrational behavior on the angiogenic hMVEC monolayer, cells were added to the hMVEC culture medium in concentrations of 1% and 10% (expressed as a percentage of the total number of hMVECs seeded on the fibrin gel) 24 hours after seeding of the hMVEC. The following cell populations were studied: CD34⁺ cell-enriched hCB-MNC (1%CD34⁺, 10%CD34⁺); CD34⁺ cell-depleted hCB-MNC (1%CD34⁻, 10%CD34⁻); and human umbilical vein endothelial cells (HUVEC) and cells from a lymphoblast cell line (Raji) as proliferating nonangioblast leukocyte-like

cell population²⁷. The hCB-MNCs were obtained from 5 donors. All experiments were performed in duplicate. The cultures were evaluated 7 days after addition of the cells to the hMVEC culture by 2 independent and blinded observers using phase-contrast microscopy. Additionally, immunohistochemical analysis of cross-sections was performed.

The movement of CD34⁺ cells on MVEC monolayers was recorded by time-lapse video phase contrast microscopy during the initial 8 hour period (1 frame per 30 seconds). The movement of individual cells was followed in time. The number of CD34⁺ cells that reached and those that remained stuck at tubular structures were counted. The number of cells that entered and remained in randomly taken comparable areas without capillary tubes were evaluated as controls.

Blocking antibodies against P-selectin (25µg/ml), E-selectin (50µg/ml), vascular cell adhesion molecule (30µg/ml), and intercellular adhesion molecule-1 (10µg/ml) were added 30 minutes before CD34⁺ cells and their effects on CD34⁺ cell accumulation at tubular structures were evaluated after 24 and 72 hours (medium was renewed after 24 hours).

Participation in new capillary formation

To investigate whether CD34⁺ cells participate in the formation of new capillary-like tubes, LNGFR-transduced CD34⁺ cells were used. LNGFR-transduced CD34⁺ cells and nontransduced CD34⁺ cells were compared for phenotypic and functional characteristics. LNGFR-transduced CD34⁺ cells were added to the hMVEC monolayer alone and together with CD34⁻ cells. After 7 days, cultures were terminated and prepared for immunohistochemical analysis.

Stimulation of new capillary formation

The effects on 3D capillary-like tube formation were studied for CD34⁺ cells alone (1% CD34⁺), CD34⁺ cells together with CD34⁻ cells (1% CD34⁺/10% CD34⁻, ie, ratio of 1:10, whereas the physiological ratio is 0.5:99.5), CD34⁻ cells alone, EPC/CAC (1%), HUVECs, and Raji cells. The effects of subpopulations of hCB-MNC on new capillary formation were assessed in 9 independent experiments (9 hCB donors). All experiments were performed in duplicate. Cell populations were added to the hMVEC culture medium 24 hours after seeding of the hMVEC monolayer. Cultures were evaluated 7 days later. Total tube length was measured as described and expressed as percentage of tube length formed by bFGF/TNF- α -stimulated hMVECs.

Immunohistochemical analysis

Fibrin matrices were fixed and embedded in paraffin. Sections (5µm) were stained for LNGFR to identify labeled CD34⁺ cells and were analyzed by light microscopy. The number of LNGFR⁺ cells containing a nucleus was counted and averaged over 5 cross-sections by a blinded observer and expressed as a percentage of the total amount of nucleus-containing cells in the monolayer.

To study proliferation of LNGFR⁺ cells *in situ*, consecutive sections were stained for LNGFR or Ki67 (expressed on all proliferating cells during late G1, S, G2, and M phases of the cell cycle)²⁸. Fibrin matrices were fixed at 4°C in 2% p-formaldehyde in PBS (pH=7.4), and embedded in paraffin. Serial sections were cut (5µm) perpendicular to the surface of the matrix sheet. Sections were deparaffinated, blocked in buffer containing 1.5% hydrogen peroxide and incubated in citrate buffer of 100°C for 20 minutes. Sections were washed and incubated with 150µl primary antibody (anti-LNGFR or anti-Ki67) for 60 minutes at 21°C. After washing sections were incubated with 150µl PowerVision goat anti-mouse poly-HRP-conjugates for 20 minutes 37°C. Diaminobenzidine was added as colouring substrate. Sections were counterstained with hematoxylin and mounted with pertex. Negative controls included substitution of the primary antibody with PBS or with an irrelevant murine IgG of the same subclass. Sections were analyzed by light microscopy. The number of LNGFR⁺ cells containing a nucleus was counted and averaged over 5 cross-sections by a blinded observer. The amount of LNGFR⁺ cells containing a nucleus was expressed as a percentage of the total amount of nucleus containing cells in the monolayer.

Immunofluorescence double-staining

Immunofluorescence double staining for LNGFR and CD31 or *Ulex europaeus* lectin was performed to confirm the endothelial phenotype of LNGFR⁺, CD34⁺ cell-derived cells in newly formed capillaries. For LNGFR/CD31 double-staining a biotin-streptavidin detection system was applied. After blocking of endogenous biotin activity, sections were incubated with 100 µl of the first biotinylated anti-LNGFR primary antibody for 60 minutes, followed by incubation with 100 µl FITC-conjugated streptavidin for 30 minutes at room temperature. To augment the signal, sections were subsequently incubated with 100 µl anti-streptavidin-FITC for 30 minutes. Remaining biotin binding sites were blocked and sections were incubated with 100 µl biotin-labeled anti-CD31 for 60 minutes followed by incubation with 100 µl TRITC-conjugated streptavidin for 30 minutes at room temperature. Sections were air-dried and mounted with Vectashield. Controls were included in which the

procedure was performed with omission of the primary antibody or substitution of the primary antibody with an irrelevant murine IgG of the same subclass.

For LNGFR/*Ulex europeus* double staining, sections were incubated in 100 µl anti-LNGFR for 30 minutes, followed by incubation with 100 µl FITC-labelled polyclonal goat anti-mouse for 30 minutes at room temperature. Subsequently, the sections were incubated in 100 µl TRITC-labelled *Ulex europeus* for 30 minutes at room temperature and air-dried and mounted with Vectashield.

Interaction between CD34⁺ and CD34⁻ cells

To investigate the interaction between CD34⁺ and CD34⁻ cells, we performed several differentiation experiments. Differentiation cultures to obtain EPC/CAC were performed as described. CD34⁺ cells were cultured in the presence or absence of CD34⁻ cells (ratio 1:100). Subsequently, cultures were performed in which CD34⁺ and CD34⁻ cells were separated by a 0.4µm pore trans-well system allowing diffusion of soluble factors without physical contact between CD34⁺ and CD34⁻ cells. Differentiation was expressed as total number of attached cells per mm² or as number of attached cells relative to the number of cultured cells.

Statistical analysis

The data are expressed as mean ± standard error of mean (SEM) unless otherwise specified. Because of variations in basal tube formation between different batches of hMVECs, stimulation of capillary-like tube formation is expressed as percentage of tube length formed by bFGF/TNF- α -stimulated hMVECs (ie, versus 'control' culture). To determine whether the different cell populations (CD34⁺, CD34⁻, CD34^{+/-} derived from the same 9 donors or EPCs, derived from 4 different donors) stimulated capillary-like tube formation significantly as compared with the reference value of 100%, 1-sample *t* tests with Bonferroni correction were performed. To compare the growth stimulating effects of CD34⁺ and CD34^{+/-} cells, which were all derived from the same 9 donors, a paired *t* test was performed. To compare the growth stimulating effect between CD34⁺ or CD34^{+/-} cells and EPC, which are derived from 4 different donors, separate independent sample *t* tests with Bonferroni correction were used. *P*<0.05 was considered statistically significant.

Results

Isolation and selection of CD34⁺ cells

Flow cytometry demonstrated that in the cell population that was enriched for CD34⁺ cells, 90.1±1.8% of cells were CD34⁺. The CD34⁻ cell population was largely depleted from CD34⁺ cells (99.8±0.1% CD34⁻ cells).

Characterization of EPC/CAC

Adherent EPC/CAC after 7 days of culture were characterized by immunohistochemistry. More than 90% of cells displayed a spindle-shaped morphology and stained positive for endothelial nitric oxide synthase, VE-cadherin, VEGFR2 (KDR), CD31, *Ulex europaeus* lectin, and DiI-acetylated LDL uptake. All controls were negative. In long-term cultures (6 weeks), clusters and confluent monolayers of cobblestone-like cells were formed (Figure 1).

CD34⁺ cells home to foci of neovascularization

hMVECs seeded on top of a fibrin matrix and cultured without addition of growth factors or cytokines remained as a quiescent monolayer on top of the matrix. When the hMVECs were exposed to both bFGF and TNF- α , they invaded the underlying fibrin matrix and formed capillary-like tubular structures (Figure 2A and 2C). When CD34⁺ cell-enriched hCB-MNC were added to the hMVEC culture medium, formation of cell clusters was observed, specifically located at the sites of new capillary-like tube formation (Figure 2G and 2H). Addition of higher concentrations of CD34⁺ cells resulted in larger cell clusters (Figure 2B versus 2D and 2E). In contrast, no such clusters were seen in the cultures to which CD34⁻ cells or HUVECs were added (data not shown). In the cultures to which cells from the Raji lymphoblast cell line were added, there was a large number of cells and cell clusters present already at day 2 (Figure 2F). However, they did not colocalize with newly formed capillary-like structures but were randomly scattered throughout the culture. Time-lapse video image microscopy during the first 8-hour period revealed that the CD34⁺ cells moved randomly over the endothelial monolayer and remained stuck at the vascular structures once they reached them (Table 1). No accumulation was seen in randomly chosen control fields of the monolayers. Viability of the homing cells was confirmed using a calcein uptake assay. Addition of antibodies against P- and E-selectin, vascular cell adhesion molecule, and intercellular adhesion molecule-1, either alone or simultaneously,

did not reduce the accumulation of CD34⁺ cells at the vascular structures (tested with 3 CD34⁺ populations from different donors) (Figure 3).

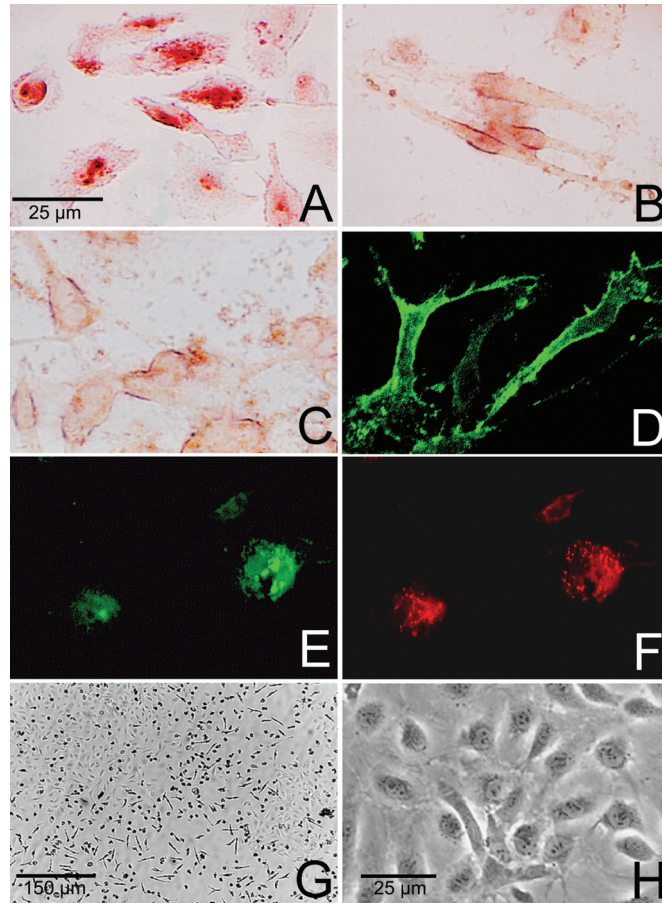


Figure 1

Characterization of EPC/CAC after 7 days of culture: expression of eNOS, perinuclear in the Golgi-system (A); VE-cadherin staining at cell-cell contacts (B); KDR staining (C); CD31 staining with typical membrane expression (D, confocal scanning laser microscopy). *Ulex europaeus* positive cells (E) take up DiI-labeled AcLDL (F) in double-staining experiments. In long-term cultures (6 weeks) formation of cell clusters and confluent monolayers of cobble stone-like cells is observed (G, H). Magnification (A-F, H) 630x; G 100x.

Table 1

	Experiment 1	Experiment 2	Cell Accumulation (% of Cells Entering the Area)
Tubular structures	13/16	27/34	80 ± 1%
Nonstructure monolayer (sum of 3 control areas):	3/56	5/130	4.6 ± 1.2 %

CD34⁺ cells accumulate specifically on endothelial tubular structures. Quantification of the accumulation of CD34⁺ cells was made by time-lapse video microscopy. CD34⁺ cells (104 cells per cm²) were added to monolayers of hMVECs that had been induced to generate tubular structures by incubation with bFGF and TNF- α . The movement of individual CD34⁺ cells over the endothelial monolayer was monitored between 2 and 8 hours after addition of the cells. To that end, 5 tubular structures in a field were encircled and the number of CD34⁺ cells that entered and left the marked areas were monitored. As controls, the surface areas of the 5 structures were projected in 3 directions to only monolayer areas without tubules or sprouting in the same microscopic field. The cell numbers of the 3 sets of control data were highly comparable and their sum is given for both experiments. Cell accumulation is given as the mean $\pm$ range.

CD34⁺ cells participate in new capillary formation

To study the contribution of CD34⁺ cells to new capillary-like tube formation, CD34⁺ cells were retrovirally transduced with the marker gene LNGFR. The CD34⁺ cell population that was genetically marked with the LNGFR gene contained 92 $\pm$ 2.6% CD34⁺ cells, which is similar to the nontransduced CD34⁺ cell-enriched mononuclear fraction. Transduction efficiency with the LNGFR gene was 39.3 $\pm$ 2.9%. After retroviral transduction with the LNGFR gene CD34⁺ cells maintained their phenotypic and functional characteristics. Addition of transduced CD34⁺ cells to hMVEC monolayers caused similar homing of cells to angiogenic sites as observed after addition of nontransduced CD34⁺ cells. Addition of transduced CD34⁺ cells to hMVEC monolayers also caused a stimulatory effect on bFGF/TNF- α -induced new capillary-like tube formation that was similar to the effect observed after addition of nontransduced CD34⁺ cells (data not shown). Immunohistochemical staining of cross-sections confirmed homing of LNGFR⁺ cells in and around new capillary sprouts and showed that LNGFR gene-marked cells participated in new capillary-like structures (Figure 4A). The transduced cells were fully integrated in the angiogenic endothelium and stained positive for the endothelial markers CD31 and *Ulex europaeus* lectin (Figure 4B to 4G). In several sections LNGFR⁺ cells were observed deep in the tubular structures, suggesting a pioneering role of CD34⁺ cells (Figure 4A and 4F). Double-staining with proliferation marker Ki67 showed that 25% to 40% of the LNGFR⁺ cells were proliferating (Figure 4H and 4I). All controls were negative.

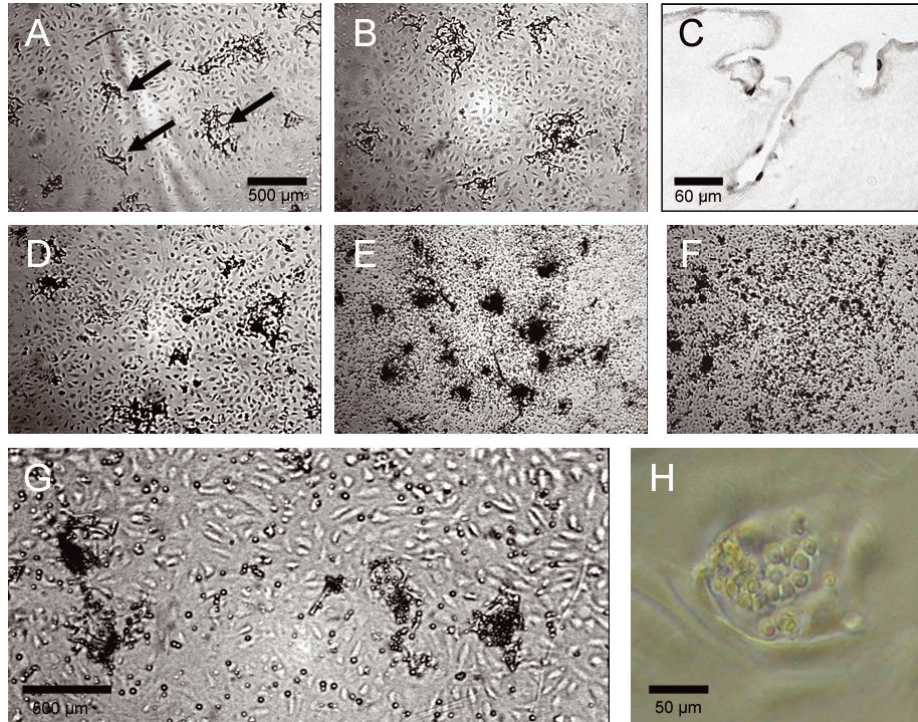
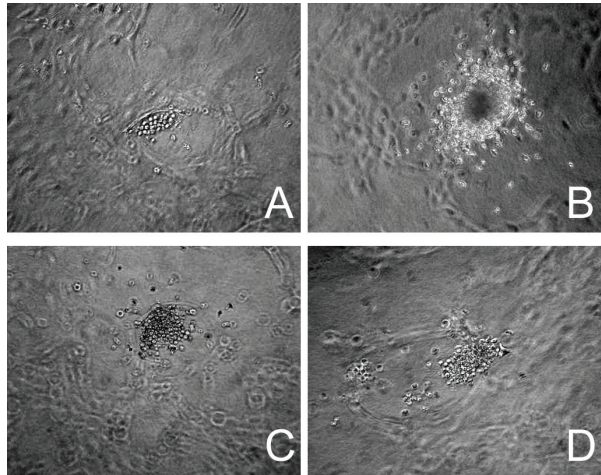


Figure 2

HMVECs were seeded at confluent density on top of a 3D fibrin matrix and stimulated with bFGF (5 ng/ml) and TNF- α (4 ng/ml). A,B,D,E Nonphase contrast photomicrographs after 7-day culture without (A) or with 1% CD34+ cells (B) or 10% CD34+ cells (D,E), which were added 24 hours after seeding the hMVECs. A, The arrows indicate sites of capillary-like tube formation by hMVEC. C, Cross-section of tubular structures at higher magnification. B,D,E, When 1% (B) or 10% CD34+ cells (D,E) were added to the hMVEC monolayer, clusters of CD34+ cells accumulated at sites of capillary-like tube formation. G, Cluster formation at angiogenic sites was visible after 4 days of culture after addition of 10% CD34+ cells (2x larger magnification). H, Early accumulated CD34+ cells at higher magnification. Addition of 1% or 10% CD34- cells or 1% or 10% HUVECs did not cause such cell clustering. F, Addition of Raji cells (10%) led rapidly to increased amounts of cells and cell clusters, which were randomly scattered throughout the culture without preference for tubular structures. Nonphase photomicrographs were taken after 7 days of culture except for (G) and (H) (phase contrast), which were taken at day 4 and 3, and (F), which was taken at day 2 because of the extensive cell proliferation and overgrowth. A-E, Data are representative photographs of 6 independent experiments using CD34+ cells from different donors. Similar results were obtained when tubular structures were induced by addition of VEGF (20 ng/mL) and TNF- α (4 ng/mL). A,B,D-F, Same magnification.

**Figure 3**

Cluster formation of CD34⁺ cells at sites of capillary-like tube formation. Culturing of cells in control medium (A); medium containing antibodies against P-selectin and E-selectin (B); medium containing antibodies against I-CAM and V-CAM (C); or medium containing antibodies against both P-selectin and E-selectin, as well as I-CAM and V-CAM (D), did not reduce CD34⁺-cell accumulation at vascular structures.

Quantification of LNGFR⁺ cell participation in capillary formation suggested that addition of CD34⁺ cells (1%) to the hMVEC monolayer led to a contribution of CD34⁺ cells of $1.0 \pm 0.5\%$ to the new vascular structures. This implies a significant incorporation of CD34⁺ cells in the endothelial lining, which was estimated $>10\%$ of the added CD34⁺ cells. The exact percentage of participation is difficult to estimate because CD34⁺ cells proliferated during the 7 days of incubation.

It should be noted that the transduction efficiency of almost 40% causes a 2.5-fold underestimation of the total amount of participating CD34⁺ cells.

CD34⁺ cells stimulate neovascularization, which is further enhanced by coculture with CD34⁻ cells

Figure 5 shows the effect of different hCB-MNC subpopulations on capillary formation in the 3D fibrin matrix. Addition of 1% EPC/CAC to the hMVEC-monolayer caused a $60 \pm 12\%$ increase in capillary formation ($p < 0.05$ versus controls). Overall, if CD34⁺ cells alone ($>90\%$ purity) were added to the hMVEC monolayer, only a slight increase in new capillary-like tube formation was observed ($17 \pm 5\%$ increase compared with controls, $p < 0.05$). When CD34⁺ cells were added in combination with CD34⁻ cells, a considerable increase of total tube length was found, which was similar to the effect of cultured EPC/CAC ($68 \pm 15\%$; $p < 0.05$). Overall, CD34⁻ cells alone did not cause a significant increase in tube length (Figure 5A). However, 2 types of responses could be distinguished in the responding cells. In preparations of 6 different donors CD34⁻ cells alone did not

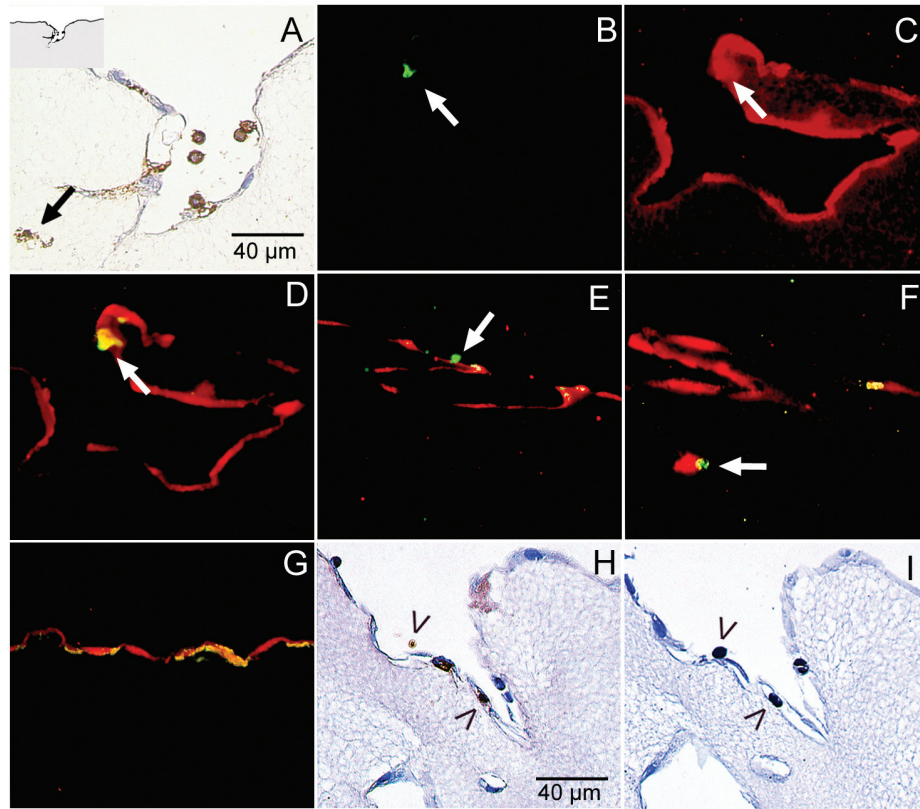


Figure 4

Histological sections showing homing, participation and differentiation of LNGFR gene-marked CD34+ cells in the capillary-like tubular structures in a fibrin matrix (400x). LNGFR gene-marked cells (LNGFR+ stained brown) home to sites of capillary formation, incorporate, become fully integrated in the endothelial cell monolayer, and acquire a phenotype that is morphologically similar to endothelial cells (A). LNGFR gene-marked cells can also be found deeper in the tubular structures in direct contact with the matrix (A, arrow). The small window within (A) gives a schematic overview. Immunofluorescent double-staining for LNGFR (green) (B) and Ulex europaeus lectin (red) (C) strongly suggests that incorporated CD34+ cells differentiate into endothelial cells (yellow) (D). Homing cells before incorporation do not stain positive for the endothelial cell marker (arrow) (E). Some of the cells at the tip of the vascular in-growth are negative for the endothelial cell marker, suggesting a higher angiogenic capacity of EPC in an earlier phase (F). Immunofluorescent double-staining for LNGFR (green) and CD31 (red) also implies endothelial differentiation of LNGFR gene-marked CD34+ cells (yellow) (G). Histological analysis of consecutive cross-sections of capillary-like tubular structures shows the presence of proliferating LNGFR gene-marked cells. H, LNGFR staining (brown). I, Corresponding consecutive cross-section, stained for Ki-67 nuclear antigen, expressed on proliferating cells (dark nuclear staining). The open arrows indicate LNGFR-Ki67 double-positive cells, indicating the presence of proliferating CD34+ cell-derived cells.

significantly increase tube length. In these preparations, stimulation of growth of vascular structures was only observed when CD34⁺ and CD34⁻ cells were added together, whereas none of the separate preparations induced a significant response (Figure 5B). In the preparations of 3 other donors the CD34⁻ cells were able to increase the extent of endothelial tube formation by themselves, which could not be further enhanced by the presence of CD34⁺ cells (Figure 5C). The latter finding suggests that in certain circumstances, for example when CD34⁻ cells are in an activated state, CD34⁻ cells may have a predominant effect on the observed tube length enhancement. Neither HUVEC nor cells from the Raji cell line significantly influenced total tube length.

Interaction between CD34⁺ and CD34⁻ cells

When isolated CD34⁺ cells were cultured alone, no differentiation into EPC occurred (0 ±0%). However, coculturing of CD34⁺ cells with CD34⁻ cells resulted in a significant increase in EPC differentiation (122±11/mm², p<0.000 versus CD34⁺ cells alone). To assess whether this increase in differentiation rate was induced by soluble growth factors, CD34⁺ cells were cultured separated from CD34⁻ cells by a membrane that allowed soluble molecules to diffuse freely but prohibited physical contact between CD34⁺ and CD34⁻ cells. EPC differentiation from CD34⁺ cells in these cultures was rarely observed (6±1/mm², p=1.0 versus CD34⁺ cells alone). Subsequently, experiments were performed in which CD34⁺ cells were labelled with PKH-26. Previous experiments showed that with this labelling method under these specific culturing conditions for a period of 7 days minimal dye transfer (<0.1%) occurred. We observed that coculturing of CD34⁺ and CD34⁻ cells led to a significant increase in differentiation of CD34⁺ cells to EPC as compared with cultures in which CD34⁺ cells were cultured alone or cultures in which CD34⁺ and CD34⁻ cells were separated using a transwell system (72±9% versus 0% or 1.5±0.3%; p<0.05). These experiments suggest that cell-cell contact between CD34⁺ and CD34⁻ cells is necessary for endothelial differentiation of CD34⁺ cells.

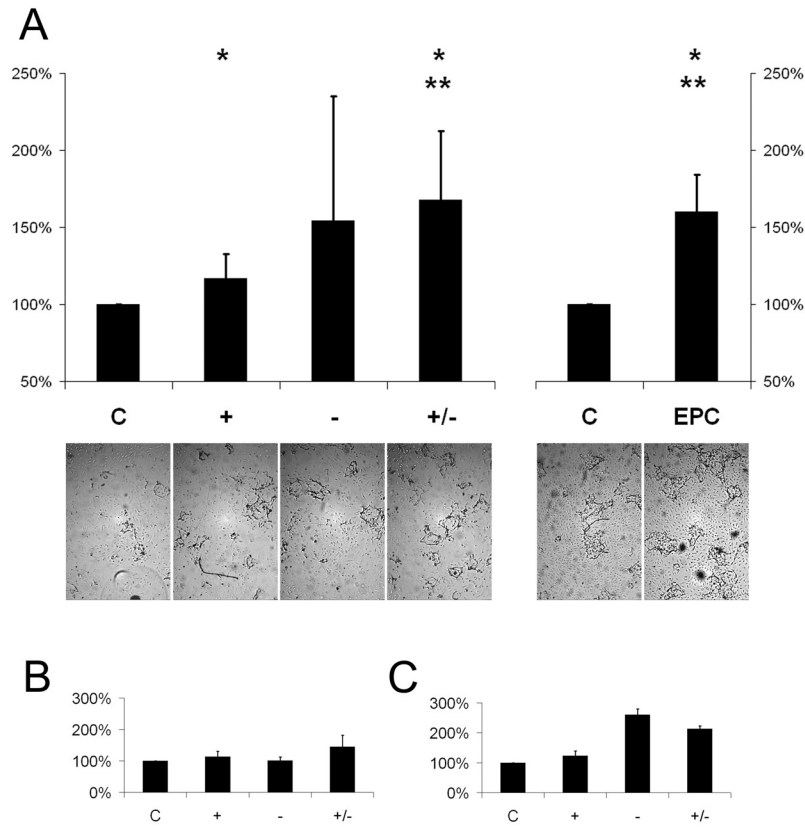


Figure 5

Effects of different subpopulations of hCB-MNC on capillary-like tube formation. The left part of (A) shows the effects of different subpopulations of hCB-MNC on capillary-like tube formation expressed as a percentage of tube length formed by bFGF/TNF- α -stimulated hMVEC. The nonphase photomicrographs show representative examples of the observed tube formation in the hMVEC monolayer in the presence of the respective hCB-MNC subpopulations. CD34⁺ cells were added to the hMVEC culture medium in a concentration of 1% either alone (+) or in combination with 10% CD34⁻ cells (+/-) and compared with control cultures (bFGF/TNF- α -stimulated hMVEC without addition of cell-populations). Data are expressed as mean $\pm$ SEM of 9 independent experiments (9 hCB donors). The right part of (A) shows the effects of hCB-derived cultured EPCs/CACs (EPC) on tube formation under the same conditions as the left panel and represent the mean $\pm$ SEM of 4 independent experiments. NS indicates nonsignificant. * Significant increase compared with controls ($p < 0.05$). ** Significant increase compared with 1%CD34⁺ stimulation ($p < 0.05$). B and C, Effects of the 2 subpopulations of the hCB-MNC given in (A) on capillary-like tube formation, namely those in which there was no significant influence on tube formation by the sole addition of the CD34⁻ cells (B; $n=6$) and the subset of the 3 CD34⁻ cell preparations that were able to increase the extent of endothelial tube formation themselves.

Discussion

In the present study, we used a recently developed *in vitro* 3D model to study different aspects of human adult vasculogenesis under controlled circumstances and demonstrate that human CD34⁺ cells selectively home to sites of angiogenic microvascular human endothelium, proliferate, become fully integrated in the microvascular endothelial monolayer, are able to differentiate into endothelial cells *in situ*, and have a mild but significant stimulatory effect on new capillary formation. Interestingly, if CD34⁺ cells are cultured on an hMVEC monolayer in the presence of CD34⁻ cells this results in marked enhancement of neovascularization similar to the effect of cultured EPC/CAC.

Although evidence is accumulating that vasculogenesis occurs in human postnatal life^{1,12,29-31}, the origin of the EPC is still under dispute. Previous studies suggested CD34⁺ cells as main source of EPC but were criticized because of CD34⁺-enriched cell populations of low purity and the possibility of vital dye transfer among cells. Our experiments, using CD34⁺ cells of high purity (>90%) and a very stable cell labeling method, ie, retroviral transduction with the marker gene LNGFR which results in permanent insertion of the LNGFR gene in the cells, strongly support a role for CD34⁺ cells as a source of EPC. Moreover, our data indicate that retroviral vectors can effectively transduce CD34⁺ cells and that transduction with a marker gene does not influence their phenotype, homing, or angiogenic capacity. Together with the ability of purified CD34⁺ cells to selectively home to angiogenic endothelium and incorporate in the endothelial monolayer, this suggests that CD34⁺ cells hold promise for gene therapy applications.

Although in our study administration of a purified population of CD34⁺ cells caused relatively modest integration of these cells in the hMVEC monolayer and only a mild stimulatory effect on neovascularization, it strongly suggests homing and incorporation of human CD34⁺ cells in human microvascular endothelium. Thus far, integration of BM cells into the endothelium has been mainly assessed in animal models. Highly variable incorporation rates have been reported, probably because of differences between models in intensity of endothelial injury. Generally, the number of incorporated endothelial cells is quite low³². Thus far, a few animal studies have assessed the effects of injection human CD34⁺ cells on neovascularization and incorporation in animal endothelium. Asahara et al were the first to report integration of DiI-labeled CD34⁺ cells in 13% or 10% of capillaries in mouse or rabbit hindlimb ischemia¹. Kocher reported incorporation of human CD34⁺ cells in 20 to 25% of total myocardial capillary vasculature in a mouse myocardial

infarction model and considerable stimulation of neovascularization resulting in improvement of cardiac function¹³. In contrast, Schatteman et al demonstrated that human CD34⁺ cells incorporated and differentiated into endothelial cells (not quantified) but did not influence restoration of blood flow in nondiabetic mice with hindlimb ischemia¹⁴. In the latter experiments administration of CD34⁺ cells did cause a marked acceleration of flow restoration in diabetic nude mice, suggesting that the effects of injected CD34⁺ cells on neovascularization may depend on (dys)function of the resident population of endothelial cells and/or angioblasts. In our model, we used a normal hMVEC population, which may already have had an optimal capillary-forming capacity. We cannot exclude the possibility that in the presence of resident endothelial cell dysfunction administration of CD34⁺ cells alone may be more effective. However, this cannot fully explain the modest stimulatory effect of CD34⁺ cells on neovascularization, as cultured EPC/CAC, administered in similar concentrations as CD34⁺ cells, did markedly enhance neovascularization in our model.

An alternative explanation for the limited effect of purified CD34⁺ cells in our *in vitro* model as compared with animal models might be the absence of (endogenous) supporting cells. Emerging evidence suggests that subsets of hematopoietic cells support capillary growth. Hematopoietic cells have been shown to release angiogenic factors such as VEGF, angiopoietins and MMPs. Additionally, subsets of recruited hematopoietic cells, particularly myelomonocytic cells, were shown to support EPC-mediated neovascularization³³. Recently, several *in vitro* studies have suggested that loco regional interactions between CD34⁺ and CD34⁻ cells may be important for proliferation and differentiation of CD34⁺ cells into endothelial cells^{1,24,34}. Interestingly, in our model homing or incorporation of CD34⁺ cells into the endothelial layer did not depend on the presence of CD34⁻ cells, suggesting that angiogenic endothelium may not only express or secrete factors to recruit CD34⁺ cells but also for their incorporation and proliferation. However, our data show that although separate administration of selected CD34⁺ or CD34⁻ cells for the majority of the donors had only limited effects on neovascularization, cocubation of both cell populations resulted in a consistent and marked increase (68%) in neovascularization, indicating that interactions between CD34⁺ cells and CD34⁻ cells can contribute to stimulation of capillary growth. Our observations that coculturing of CD34⁺ cells with CD34⁻ cells significantly enhances EPC differentiation *in vitro*, suggest that this is at least partly caused by enhanced differentiation and incorporation of CD34⁺ cells.

Apart from (CD34⁻ cell-stimulated) CD34⁺ cell incorporation enhanced capillary growth after CD34⁺/⁻ cell cocubation may be explained by a stimulatory effect of CD34⁺ cells on

endothelial differentiation of CD34⁺ cells. Several studies have demonstrated that CD34⁺ cells may differentiate into endothelial cells *in vitro*^{19,20,35}. CD34⁺ cells may even be the main source of cultured EPCs²¹. Several studies reported only rare incorporation of CD34⁺ cells into new capillaries *in vivo*^{1,14} without functional improvements in blood flow. It has been suggested that CD34⁺ and CD14⁺ cells yield the 'early EPC,' which have no significant proliferative capacity and contribute to neovascularization mainly by secreting angiogenic cytokines^{20,22}. However, Harraz et al¹⁵ demonstrated that CD34⁺ cells and CD34⁺/CD14⁺ cells may incorporate into the neovasculature *in vivo* when coadministered with CD34⁺ cells. Such potential integration of CD34⁺ cells is intriguing and warrants further study. However, our study was not designed to demonstrate or exclude endothelial differentiation or incorporation of CD34⁺ cells. The aim of our present study was to investigate specifically the role of human CD34⁺ cells in human neovascularization. We chose to use retroviral transduction with a marker gene in our CD34⁺ cells because this is a very stable and reliable labeling method and because retrovirally transduced autologous CD34⁺ cells have already been applied for clinical use. This labeling method is, however, not suitable for labeling of the CD34⁺ cell population.

It should be noted that our study is confined to the formation of human capillary-like tubular structures *in vitro*. However, if confirmed, our findings have important clinical and therapeutic consequences. They suggest that administration of a CD34⁺-enriched cell population may lead to a significant improvement of neovascularization, which is similar to the effects observed with cultured EPC/CAC. The use of CD34⁺-enriched cells has the advantage that fresh administration of these cells after brief manipulation is much easier than culturing EPCs, which involves prolonged sterile manipulation. Furthermore, if cultured EPCs/CACs are indeed monocytes/macrophages, potentially harmful effects caused by their proinflammatory characteristics have to be taken into account. Finally, much experience has been obtained over the years as CD34⁺ cell-enriched cell populations have been used extensively for treatment of hematological and nonhematological malignancies and *ex vivo* expansion methods have been developed^{23,36,37}. Our observation that human CD34⁺ cells selectively home and incorporate in angiogenic endothelium suggests their potential for gene therapy. At last, our observations suggest an important supportive role for the CD34⁺ cell population. Further studies should be aimed at unraveling the interactions between CD34⁺ and CD34⁺ cells.

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