$CD34^+$ 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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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. (***Arterioscler Thromb Vasc Biol*. 2005;25:1843-1850.)**

Key Words: angiogenesis ■ nitric oxide, endothelium, vascular type ■ gene therapy ■ peripheral vascular disease

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,[1] a process that, reminiscent of embryonic vessel formation, is termed adult vasculogenesis.[2] 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 have 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 reported.[3–6]

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 atherosclerosis,[7] 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.

Original received December 29, 2003; final version accepted July 1, 2005.

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000177808.92494.14**

Several studies have suggested that in human postnatal life, analogous to the existence of the embryonic hemangioblast,[10] 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 lacking.[15] Several investigators pointed at a more important role for the $CD34^-CD14^+$ monocytic cell population.[15–19] Interestingly, recent reports suggest that the majority of blood-derived cultured acetylated low-density lipoprotein (LDL) and *Ulex europeus* double-positive cells, which are commonly referred to as EPCs, may be derived from monocytes/macrophages.[20] We previously demonstrated that a minor but significant subset (≈9%) of cultured EPC originates from the $CD34^+$ mononuclear cell population.[21] 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 potency.[20–22]

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

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 (for detailed description, see http://atvb.ahajournals.org). 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 previously[23] 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 (for detailed description, see http://atvb.ahajournals.org).

Differentiation and Characterization of EPC/CAC

EPC/CAC were obtained as previously described[24] by culturing hCB-MNC at a density of 2×10^6 cells/cm^2 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 endothelial nitric oxide synthase, vascular endothelial (VE)-cadherin, kinase insert domain receptor (KDR), CD31, *Ulex europeus* agglutinin-I, and the ability to take up acetylated LDL (for detailed description, see http://atvb.ahajournals.org).

In Vitro Neovascularization Assay

In vitro neovascularization assays were performed in human fibrin matrices as described previously.[25] In short, highly confluent hMVECs (0.7×10^5 cells/cm^2) were seeded in a 1.25:1 split ratio on fibrin matrices (for detailed description, see http://atvb.ahajournals.org) 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 mm^2 per field) per well were analyzed and used to calculate the total length of the tube-like structures, expressed as mm/cm^2.

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 (HUVECs) and cells from a lymphoblast cell line (Raji) as proliferating nonangioblast leukocyte-like cell population.[26] 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 hours 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, E-selectin, vascular cell adhesion molecule, and intercellular adhesion molecule-1 (for details see http://atvb.ahajournals.org) 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 (5 μm) 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[27]) (for detailed description, see http://atvb.ahajournals.org).

Immunofluorescence Double-Staining

Immunofluorescence double-staining for LNGFR and CD31 or *Ulex europeus* lectin was performed to confirm the endothelial phenotype of $LNGFR^+$, $CD34^+$ cell-derived cells in newly formed capillaries (for detailed description, see http://atvb.ahajournals.org).

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 0.4-μm 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 mm^2 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/TNFα-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 EPCs that 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 europeus* 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 (for detailed description, see http://atvb.ahajournals.org).

$CD34^+$ 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 1A and 1C). 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 1G and 1H). Addition of higher concentrations of $CD34^+$ cells resulted in larger cell clusters (Figure 1B versus 1D and 1E). 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 1F). 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). 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; for detailed description, see http://atvb.ahajournals.org).

$CD34^+$ 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 underwent genetic marking with the LNGFR gene contained 92±2.6% $CD34^+$ cells, which is similar to the nontransduced $CD34^+$ cell–enriched mononuclear fraction.

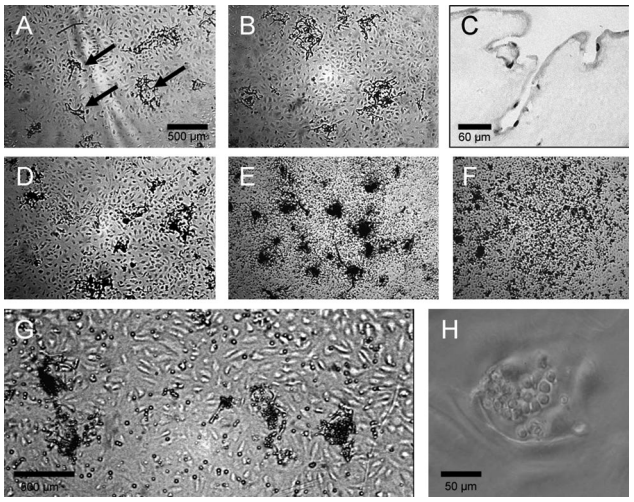

Figure 1. HMVECs were seeded at confluent density on top of a 3D fibrin matrix and stimulated with bFGF (5 ng/mL) and TNF-α (4 ng/mL). A,B,D,E, Nonphase contrast photmicrographs after 7-day culture without (A) or with 1% $CD34^+$ cells (B) or 10% $CD34^+$ cells (D,E), which were added 24 hours after 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 (2× 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 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 days 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.

Transduction efficiency with the LNGFR gene was 39.3±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 2A). The transduced cells were fully integrated in the angiogenic endothelium and stained positive for the endothelial markers CD31 and *Ulex europeus* lectin (Figure 2B to 2G). In several sections, $LNGFR^+$ cells were observed deep in the tubular structures, suggesting a pioneering role of $CD34^+$ cells

$CD34^+$ Cells Accumulate Specifically on Endothelial Tubular Structures

	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%

Quantification of the accumulation of $CD34^+$ cells was made by time-lapse video microscopy. $CD34^+$ cells (10^4 cells per cm^2) 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±range.

(Figure 2A and 2F). Double-staining with proliferation marker Ki67 showed that 25% to 40% of the $LNGFR^+$ cells were proliferating (Figure 2H and 2I). All controls were negative.

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±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 3 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±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±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±15%; $P<0.05$). Overall, $CD34^-$ cells alone did not cause a significant increase in tube length (Figure 3A). However, 2 types of responses could be distinguished in the responding cells. In preparations of 6 different donors $CD34^-$ cells alone did not significantly increase tube length. In these preparations, stimulation of growth of vascular structures was only

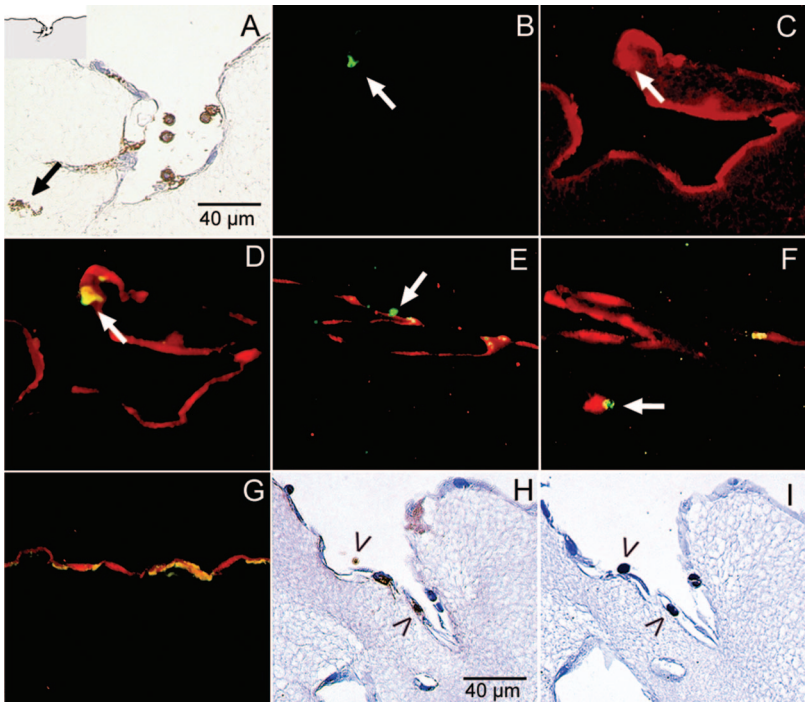

Figure 2. Histological sections showing homing, participation, and differentiation of LNGFR gene–marked CD34$^+$ cells in the capillary-like tubular structures in a fibrin matrix (400×). LNGFR gene–marked cells (LNGFR$^+$ stained brown) home to sites of capillary formation, incorporate, become fully 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 europeus 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.

observed when CD34$^+$ and CD34$^-$ cells were added together, whereas none of the separate preparations induced a significant response (Figure 3B). 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 3C). 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

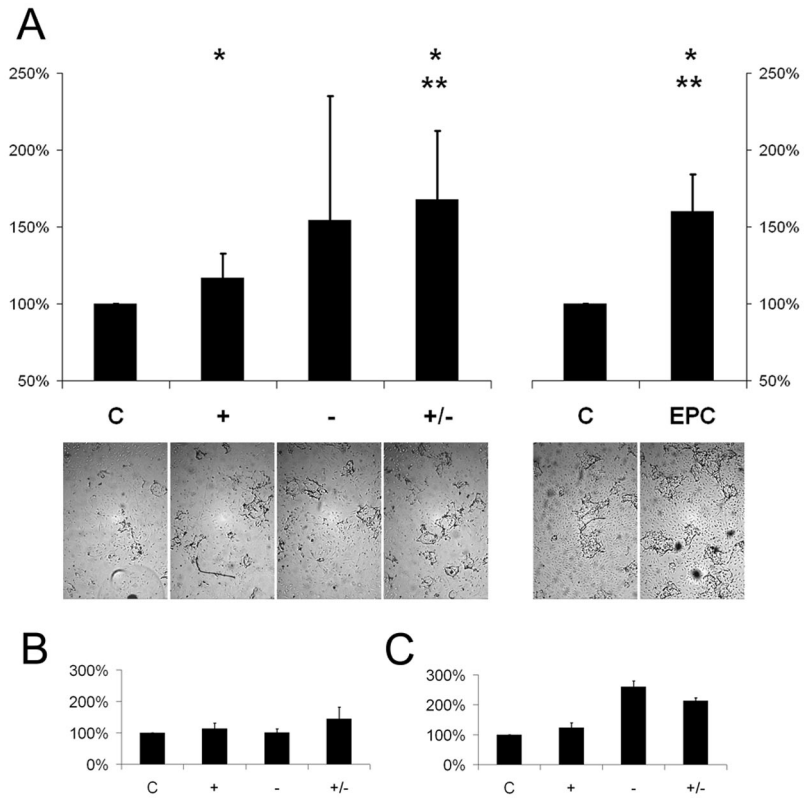

Figure 3. Effects of different subpopulations of hCB-MNC on capillary-like tube formation. The left part of (A) shows the effects of different subpopulations of hCB-MNC on capillary-like tube formation expressed as a percentage of tube length formed by bFGF/TNF-α–stimulated hMVEC. The nonphase photomicrographs 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±SEM of 9 independent experiments (9 hCB donors). The right part of (A) shows the effects of hCB-derived cultured EPCs/CACs (EPCs) on tube formation under the same conditions as the left panel and represent the mean±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.

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/mm^2, $P<0.000$ 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/mm^2, $P=1.0$ versus CD34$^+$ cells alone). Subsequently, experiments were performed in which CD34$^+$ cells were labeled with PKH-26. Previous experiments showed that with this labeling 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.

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,[1,11,28–30] 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 low.[31] 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 ischemia.[1] 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 function.[13] 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 ischemia.[14] 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 neovascularization.[32] Recently, several in vitro studies have suggested that locoregional interactions between CD34$^+$ and CD34$^-$ cells may be important for proliferation and differentiation of CD34$^+$ cells into endothelial cells.[1,24,33] 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, coincubation 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 coincubation 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.[18,20,34] $CD34^-$ cells may even be the main source of cultured EPCs.[21] 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 al[15] 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 hematologic and nonhematologic malignancies and ex vivo expansion methods have been developed.[23,35,36] 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.

Acknowledgments

M.C.V. is supported by NWO (VENI grant 016.036.041). This work was financially supported by the Dutch Kidney Foundation (grant PC 127) and the Netherlands Heart Foundation (NHF grant 2002B157).

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