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Myoendothelial Differentiation of Human Umbilical Cord Blood–Derived Stem Cells in Ischemic Limb Tissues

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Abstract—Human umbilical cord blood (UCB) contains high numbers of endothelial progenitors cells (EPCs) characterized by coexpression of CD34 and CD133 markers. Prior studies have shown that $CD34^+/CD133^+$ EPCs from the cord or peripheral blood (PB) can give rise to endothelial cells and induce angiogenesis in ischemic tissues. In the present study, it is shown that freshly isolated human cord blood $CD34^+$ cells injected into ischemic adductor muscles gave rise to endothelial and, unexpectedly, to skeletal muscle cells in mice. In fact, the treated limbs exhibited enhanced arteriole length density and regenerating muscle fiber density. Under similar experimental conditions, $CD34^-$ cells did not enhance the formation of new arterioles and regenerating muscle fibers. In nonischemic limbs $CD34^+$ cells increased arteriole length density but did not promote formation of new muscle fibers. Endothelial and myogenic differentiation ability was maintained in $CD34^+$ cells after ex vivo expansion. Myogenic conversion of human cord blood $CD34^+$ cells was also observed in vitro by coculture onto mouse myoblasts. These results show that human cord blood $CD34^+$ cells differentiate into endothelial and skeletal muscle cells, thus providing an indication of human EPCs plasticity. The full text of this article is available online at http://www.circresaha.org. **(*Circ Res*. 2003;93:e51-e62.)**

Key Words: stem cells ■ CD34 ■ *trans*-differentiation ■ angiogenesis ■ myogenesis

Endothelial progenitors cells (EPCs) were originally identified as a scant population of stem cells in human peripheral blood (PB), characterized by expression of CD34, vascular endothelial growth factor receptor (VEGFR2, KDR), and CD133 markers.[1,2] The number of circulating EPCs in the peripheral blood is normally very low, representing less than 0.1% of the total mononuclear fraction of human PB. However, vascular trauma or ischemia have been reported to increase the number of these cells in the blood stream.[2,3] In humans, cells displaying hematopoietic (HSC) stem cell activity have also been found in umbilical cord blood (UCB) where their number is higher compared with normal PB.[4] UCB progenitor cells are routinely used in patients affected by major hematological disorders as an alternative to bone marrow transplantation for stem cell reconstitution.[5] It has been shown that $CD34^+$ cells obtained from human PB or UCB give rise to mature endothelial cells when cultured onto specific substrates or by stimulation with growth factors in culture.[1,3] Furthermore, it has been described the ability of these cells to improve neovascularization and to increase blood flow in immunodeficient animal models of hind limb ischemia.[3,4,6–8] EPCs have now become clinically relevant, as trials of therapeutic angiogenesis based on autologous bone marrow cell transplantation in patients affected by severe limb or myocardial ischemia have been recently published.[9–12]

In the present study, we show that UCB $CD34^+$ cells survive and colonize tissues of Cyclosporine-A (Cs-A)–immunosuppressed mice. Analogous to previous observations using human UCB cells or bone marrow cells,[1,4,13–15] we report that these cells undergo a program of endothelial differentiation and promote the formation of new blood vessels. In addition, we challenge the concept that UCB stem cells are a committed pool of hematopoietic/endothelial stem cells. In fact, we found that UCB stem cells improve muscle fibers regeneration by differentiating into myogenic cells. We extended this last observation by coculturing $CD34^+$ cells onto mouse myoblast feeder layers. Under these conditions, $CD34^+$ cells formed myotubes in mixed cultures, thus showing that these cells can be driven toward endothelial or myogenic differentiation pathways depending on culture conditions. Altogether, these findings suggest that UCB-derived stem cells are a multipotent stem cell population displaying wider differentiation plasticity than previously supposed and provide an indication of myogenic conversion of human EPCs.

Materials and Methods

Isolation and Culture of EPCs From UCB

Placental blood was recovered in EDTA-containing bags immediately after delivery. UCB collection was performed with written approval by mothers. The age of neonates ranged between 36 and 42 weeks of gestation. Isolation of $CD34^+$ cells was performed by

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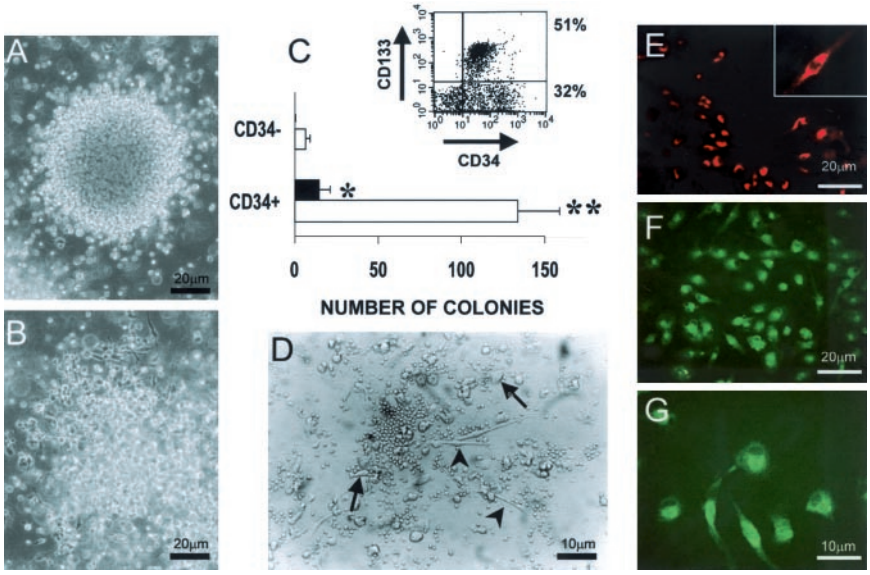

Figure 1. Endothelial differentiation of UCB CD34$^+$ cells. Methylcellulose culture of CD34$^+$ and CD34$^-$ MACS fractions. A, Typical morphology of a CFU-GM colony resulting from clonal expansion of HSCs within the CD34$^+$ cell fraction. B, Representative CFU-EC colony composed of dispersed cells that partially adhered to the culture dish.[7] These cells were subcultured in liquid culture and tested for uptake of Ac-LDL-DiI revealing endothelial phenotype (not shown). C, Quantitative determination of the hematopoietic colonies (CFU-GM, CFU-M, CFU-G) and CFU-EC colonies obtained from culturing CD34$^+$ and CD34$^-$ cells in methylcellulose. CD34$^+$ cells produced significantly higher numbers of hematopoietic progenitor cell colonies (open bar) and CFU-EC (filled bar) than CD34$^-$ cells (n=4). Inset shows a typical FACS profile of MACS-sorted CD34$^+$ cells analyzed for CD34 and CD133 antigens. Percentages of CD34$^+$ and double CD34$^+$/CD133$^+$ cells are indicated. *$P<0.05$ and **$P<0.05$ (*t* test) for each colony type obtained from CD34$^+$ compared with CD34$^-$ cells. D, Liquid culture of UCB CD34$^+$ onto FN-coated dishes. Round floating or weakly attached cells (arrows) and adherent cells (arrowheads) were observed after 7 days of culture. E, UCB CD34$^+$-derived cells in liquid culture take up Ac-LDL-DiI. Inset shows a higher magnification of a spindle-like cell positive for AC-LDL-DiI uptake. F, Immunohistochemistry of UCB CD34$^+$-derived cells using anti–Von Willebrand antibody. G, Immunohistochemistry of UCB CD34$^+$-derived cells using anti-KDR receptor antibody. Intracellular staining for KDR receptor was likely due to the permeabilization protocol necessary for unmasking the intracellular epitope recognized by the antibody (see Materials and Methods).

MINIMACS system using the direct CD34 isolation kit (Miltenyi Biotech, catalog No. 130-046-702) according to manufacturer's instructions, after obtaining the UCB mononuclear fraction by Ficoll-Histopaque (Sigma Immunochemicals) gradient separation. Purity of sorted cells was approximately 70% to 80% as assessed by FACS analysis using anti-CD34 and anti-CD133 antibodies labeled with phycoerythrin (PE) or fluorescein (FITC) (Figure 1C). For endothelial differentiation, MACS-sorted cells were cultured onto Laboratory-Tek chambers slides coated either with 0.1% gelatin or 20 μg/mL fibronectin (FN) in RPMI medium containing 20% FCS and processed for AC-LDL-Di uptake (10 μg/mL in culture medium for 4 hours) or immunohistochemistry after 10 days. For methylcellulose cultures, 10^4 CD34$^+$ or CD34$^-$ per dish cells were plated in triplicate in methylcellulose medium (Stem Cells Technologies, catalog No. GF$^+$H4535) containing interleukin-6 and -3 (IL-6, IL-3), stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) cytokines. After 14 days, the numbers of colonies containing granulocyte/macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), and endothelial cells (CFU-EC)[7] were counted. To evaluate the expansion of stem cell–derived hematopoietic colonies, we grouped together CFU-GM, CFU-G, and CFU-M colonies as representative of the colony forming activity by clonogenic cells in both the CD34$^+$ and CD34$^-$ MACS fractions. For coculture onto myoblasts or injection into ischemic tissues, CD34$^+$ and CD34$^-$ cells were labeled with 1-1′-dioctadecyl-3-3-3′-3′-tertamethylindocarbocyanine-perchlorate (DiI) living dye[1] at 5 μg/mL in serum-free RPMI medium for 30 minutes. After washing, cells were seeded onto a 60% confluent layer of C_2C_{12} myoblasts for 2 days in proliferation medium (DMEM containing 20% FCS) before starving in differentiation medium (DMEM containing 2% horse serum) for 4 days. Under these conditions, before serum starvation, CD34$^+$ cells attached over myoblasts whereas, after starvation, they fused within differentiating myotubes. In some experiments, C_2C_{12} cells were irradiated with 10 Gy X-rays.[16] Transduction of CD34$^+$ cells and C_2C_{12} mouse myoblast cell line with a retrovirus carrying enhanced green fluorescent protein (EGFP) was performed by incubating cells with the viral supernatant produced by Phoenix packaging cell line stable transfected with pPINCO retroviral vector,[17] according to instructions described at http://www.stanford.edu/group/nolan/retroviral_systems/phx.html. Transduction efficiency ranged between 20% to 30% as evaluated by fluorescence microscope cell counting of five different fields/experiment containing freshly transduced cells.

Expansion of CD34$^+$ cells was performed using a serum-free medium (Stem Span, Stem cell Technologies) supplemented with 100 ng/mL Flt3-ligand, 100 ng/mL SCF, 20 ng/mL IL3, 20 ng/mL IL6 (all by Stem cell Technologies). After 7 days in culture, cells were counted and analyzed for CD34, CD133, and CD45 antigen expression by FACS analysis.

FACS Analysis

FACS analysis of MACS-sorted cells or CD34$^+$ cells after expansion in culture was performed by incubating cells with 1 μg/mL FITC- or PE-conjugated mouse anti-human CD34 and CD133 monoclonal antibodies (BD-Pharmingen) for 20 minutes at 4°C in PBS containing 5% FCS. PE- or FITC-conjugated mouse IgGs were used as isotype control in FACS analysis at the same concentration as specific primary antibodies; 10^4 cells with no gate were analyzed in each sample into a FACScalibur fluorescence activated cell sorter (Beckton-Dickinson).

Animals and Surgical Procedures

Swiss CD1 male mice, 2 months old (Charles River, Italy), were used in this study. Immunosuppression was performed by injecting Cs-A at 20 mg/kg weight for 2 days before, and daily after the surgery, for the entire period of the experiment. To produce hind limb ischemia, the left femoral artery in anesthetized mice (2,2,2-tribromoethanol, 880 mmol/kg body weight, Sigma Immunochemicals) was excised with an electrocoagulator from its proximal origin as a branch of the external iliac artery till the bifurcation into saphenous and popliteal arteries as described.[18] Injection of human EPCs was performed at the same time by injecting cells resuspended in 30 μL PBS at three levels (proximal, medial, distal, 10 μL each) of the adductor muscle, along the femoral artery site after its removal.

Histology

For cryosectioning, unfixed adductor muscles were removed 7 or 14 days after ischemia and immediately frozen in isopentane in liquid

N_2 for later inclusion in OCT compound (Bio Optica) and sectioning at 5 μm into a cryostat. For histology, mice were perfused first with 50 mL phosphate buffer (0.2 mol/L, pH 7.4) containing 5000 U/mL heparin (Roche) and then with 4% paraformaldehyde in PBS (pH 7.4) for 10 minutes. The dissected adductor muscles were embedded in paraffin and sectioned at 3 μm. Hematoxylin-eosin staining was performed in order to count muscle fibers, whereas immunohistochemistry using anti–α-actin antibody was performed to quantify arteriole length density (see next section).

Immunohistochemistry

Endothelial cells obtained in culture from $CD34^+$ cells were analyzed by immunohistochemistry using a mouse monoclonal anti–Flk-1/KDR (clone A3, Santa Cruz) at 1 μg/mL in PBS containing 1 mg/mL BSA and 0.1% Triton X-100 (TX), followed by incubation with FITC-conjugated anti-mouse antibodies in the same medium. Factor VIII (Von Willebrand factor) immunohistochemistry was performed by incubating the cells with a rabbit polyclonal antibody (DAKO) at 10 μg/mL in TX-containing PBS-BSA followed by incubation with FITC-conjugated, anti rabbit polyclonal antibodies. Frozen section of adductor muscles from mice injected with DiI-labeled UCB $CD34^+$ cells were first fixed by incubating with 2% paraformaldehyde in PBS for 15 minutes at room temperature and then incubated with 1 to 10 μg/mL mouse monoclonal anti-desmin (clone DE-U-10, Sigma Immunochemicals), mouse monoclonal anti–α-actin (clone 1A4, Sigma Immunochemicals) or rabbit polyclonal anti laminin (Sigma Immunochemicals) antibodies in PBS containing 2% BSA. After washing, sections were stained with fluorescein-conjugated goat anti-mouse or goat anti-rabbit polyclonal antibody (DAKO) in the same medium. Human MyoD staining was performed with a specific mouse anti-human MyoD monoclonal antibody (clone G106-647, BD-Pharmingen) at 2.5 μg/mL concentration in PBS containing BSA. A biotinylated monoclonal anti-mouse antibody was used as a secondary antibody, followed by ABC complex incubation and DAB Peroxidase staining kit (Vector Laboratories). For GFP immunohistochemistry, sections were incubated with anti-GFP rabbit polyclonal antibody (AbCam, No. Ab290) at a 10 μg/mL concentration, followed by FITC-conjugated anti-rabbit antibodies. For arteriole counting, paraffin sections of adductor muscles were stained with anti-mouse smooth muscle α-actin (see before) and further stained with rhodamine-conjugated goat anti-mouse polyclonal antibody (Sigma Immunochemicals). Sections were observed under a ZEISS Axiovert fluorescence microscope and images were acquired and stored with image analyzer KS300 software.

Morphometric Analysis

The method for evaluating the arteriole length density has been described previously.[19,20] Briefly, sections of adductor muscles stained with anti–α-actin from each mouse were examined under fluorescence microscope. The measures of major and minor diameters of each arteriole along with the arteriole wall thickness were determined and scored by KS300 imaging software. For "n" arterioles scored in a given area (A), the length density (Ld) corresponds to the sum of the ratios (R_n) between the major and the minor axes of each arteriole. Thus, Ld is equal to the length per unit volume in the same dimensional area: $Ld = 1/A\Sigma R$, where R is $(R_1+R_2+.....+R_n)$. The number of regenerating muscle fibers in the adductor muscles of saline- or cell-injected mice was calculated by counting the total number of fibers that showed centrally located nuclei.[21] This number was normalized to the section area calculated with KS300 imaging software. Sections showing infiltration by T lymphocytes, likely due to rejection of human cells (Figure 2A), were not included in morphometric evaluations. All mice displaying rejection associated with necrosis of the tissues (Table) were not considered for morphometric determination of arteriole length density and muscle fiber density.

Statistical Analysis

Cell culture experiments and FACS analyses were performed in triplicate in at least three different experiments. Significance was calculated by Student's *t* test using Jandel Sigma-Stat statistical software. A $P<0.05$ was taken to indicate statistical significance. Results are reported as average±SE.

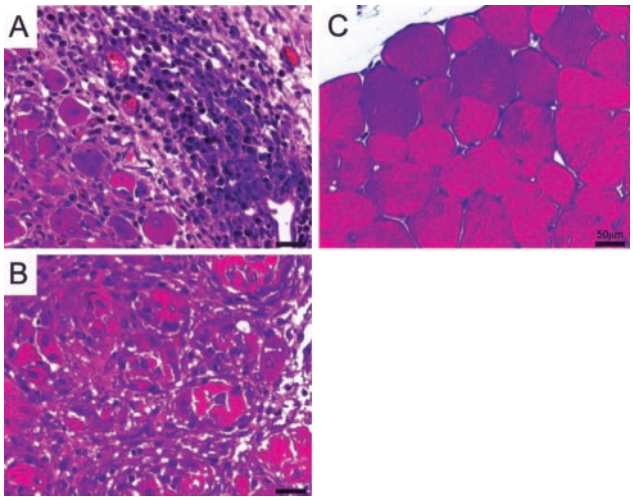

Figure 2. Assessment of rejection in mice injected with human stem cells and morphology of necrotic tissues as a consequence of ischemia. A, Acute inflammation of tissues was assessed by the presence of numerous T lymphocytes that infiltrated the tissue. B, Morphology of adductor muscles in mice that showed signs of massive tissue degeneration after ischemia. Note swollen muscle fibers and the presence of numerous granulocytes and neutrophils indicative of tissue inflammation. C, Morphology of normoperfused adductor muscle.

Results

Differentiation of UCB $CD34^+$ Cells Into Endothelial Cells In Vitro

$CD34^+$ cell separation from the mononuclear fraction of UCB was performed using a sorting approach based on magnetic separation (see Materials and Methods). Enrichment of EPCs in the $CD34^+$ cell fraction was evaluated by FACS analysis of CD34 and CD133 marker expression. Inset in Figure 1C shows a typical FACS profile of single $CD34^+$ and double $CD34^+/CD133^+$ cells (total $CD34^+$ cells: 83%). Expansion in methylcellulose medium containing cytokines was performed to confirm that the $CD34^+$-enriched populations contained cells having the potential to differentiate into hematopoietic and endothelial cells in vitro. In these conditions, $CD34^+$ cells gave rise to hematopoietic colonies (CFU-GM, CFU-M, and CFU-G; Figure 1A) and colonies containing endothelial cells (CFU-EC; Figure 1B),[7] whereas the $CD34^-$ cells produced only a few colonies (Figure 1C). To show the ability of $CD34^+$ cells to differentiate into endothelial cells, we performed liquid culture of enriched cells obtained by CFU-EC colonies grown in methylcellulose or freshly isolated UCB $CD34^+$ cells onto gelatin or fibronectin-coated dishes in the presence of 20% FCS.[1] In these conditions, the cells attached and spread onto the substrate and, after 7 days of culture, formed clusters of spindle-shaped cells with typical endothelial cell morphology (Figure 1D). These cells were tested for the uptake of Ac-LDL-DI substrate (Figure 1E)[1] and for the expression of endothelial cells markers, ie, Von Willebrand factor (Figure 1F) and KDR receptor (Figure 1G).[22,23]

Infiltration in Mouse Adductor Muscles After Injection With the Indicated Cells or Saline

Treatment	Time	Total No. of Mice	No. of Mice With Infiltration	Percentage of Mice With Infiltration
$CD34^+$	Day 7	9	1	11.1
	Day 14	4	1	25
$CD34^-$	Day 7	3	0	0
	Day 14	6	3	50
Expanded $CD34^+$	Day 7	4	1	25
	Day 14	4	1	25
Saline	Day 7	6	0	0
	Day 14	4	0	0
$CD34^+$ no ischemia	Day 7	3	0	0
$CD34^-$ no ischemia	Day 7	3	0	0
Total mice injected with human cells	Day 7	22	2	9.1
	Day 14	14	5	35.7

Percentages to the total number of mice analyzed at each time point are indicated.

Injection of Human Stem Cells Into Ischemic Limbs in Immunosuppressed Mice

Previously, it has been shown in NOD/SCID immunodeficient mice[1,13] or nude rats[4] that EPCs from PB or UCB promote angiogenesis in vivo. To date, however, no studies of this type have been performed using immunosuppression protocols of normal animals. Therefore, 1×10^5 purified UCB $CD34^+$, $CD34^-$ cells, or saline were injected into immunocompetent Swiss CD-1 mice treated with Cs-A for 2 days before, and daily after the injection of stem cells in the adductor muscle of ischemic limbs. Injection was performed at three different levels (proximal, medial, and distal) in the adductor muscle along the site of femoral artery after its removal, in a way that cells became distributed into the ischemic muscle. To evaluate rejection of injected human cells, histological sections of adductor muscles of each mouse were examined at three different levels (proximal, medial, and distal) to search for generalized inflammation of injected tissues. At 7 days after injection, only 2 mice out of 22 injected with human cells (9.1%) exhibited histological evidence of an acute inflammation (Table and Figure 2A), distinct from ischemia-induced tissue necrosis (Figure 2B) or normoperfused tissue morphology (Figure 2C). At 14 days after injection, the number of mice displaying inflammation in the adductor muscle raised to a 5:14 ratio (35.7%). This was also associated with necrosis, indicating that immunosuppression protocol failed to prevent all mice from developing a chronic rejection of human cells or graft versus host disease (GVDH).

Endothelial Differentiation Potential of UCB $CD34^+$ Cells In Vivo

To measure the overall angiogenic effect of injected cells, we determined the length density of arterioles 4 to 41 μm in diameter[24] (Figure 3A) in adductor muscles of mice injected with $CD34^+$, $CD34^-$, and saline, 7 and 14 days after ischemia. As shown in Figure 3B, the arteriole length density in ischemic limbs was significantly increased at both time points by $CD34^+$ cells, whereas $CD34^-$ cells did not induce an angiogenic response compared with saline-treated mice, except for a slight increase at 14 days that, however, was significantly lower than in mice injected with $CD34^+$ cells. To determine whether injection of stem cells contributed to angiogenesis in vivo in the absence of ischemia, UCB $CD34^+$ cells were injected into nonischemic adductor muscles and arteriole length density was determined after 7 days. The results showed that $CD34^+$ cells enhanced the number of arterioles also in nonischemic muscles to a level comparable to that achieved in ischemic condition, whereas $CD34^-$ cells did not have such potential.

To allow recognition of injected human cells in mouse tissues, UCB $CD34^+$ cells were infected with a retrovirus carrying EGFP-protein[17] before being injected in the ischemic muscles.[1] Transduction efficiency of these cells ranged between 20% and 30%, as evaluated by fluorescence microscope examination of living cells (not shown, see Materials and Methods). Thereafter, injected cells in recipient tissues were identified by immunohistochemistry for GFP in fluorescence microscopy analysis of muscle cross sections. We found that a number of arterioles contained GFP^+ cells (Figure 3C). To quantitatively measure the contribution of UCB $CD34^+$ cells to endothelial cells (ECs) formation in stem cell injected mice, GFP^+ endothelial cells were counted in adductor muscle sections triple stained with α-actin antibodies, GFP antibodies and Hoechst nuclear dye. We calculated the average number of GFP^+ EC cells compared with the average number of total EC cells in the same arterioles. The results of cell counting performed in a total of 97 arterioles (range 11 to 41 μm) in 3 different mice injected with GFP^+ $CD34^+$ cells revealed an average of 1.66 ± 0.21 GFP^+ ECs/arteriole in a total of 5.85 ± 1.22 ECs/arteriole (29%). It has been recently reported that peripheral blood contains precursors of vascular smooth muscle cells (VSMCs).[25,26] To investigate whether injected UCB cells differentiated into VSMCs, we screened for double GFP^+/α-$actin^+$ cells in the arteriole wall (Figure 3E). GFP^+/α-$actin^+$ cells were sporadically noticed into the arteriole wall suggest-

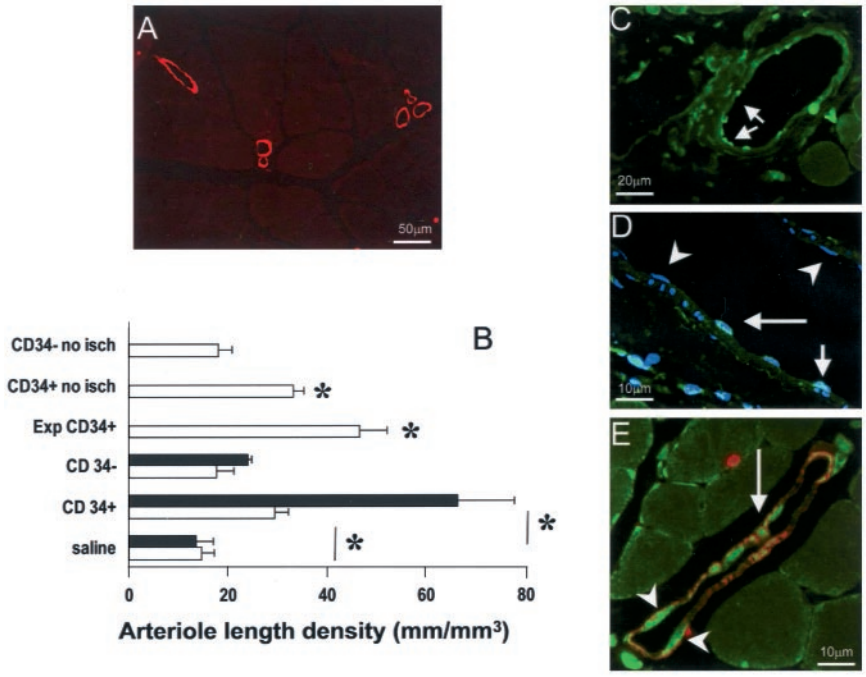

Figure 3. Injection of CD34+ cells enhances arteriole length density in ischemic adductor muscles. A, Immunohistochemistry for α-actin in a paraffin section of adductor muscle for arteriole length-density evaluation. B, Average arteriole length density in ischemic limbs. Injection of UCB CD34+ cells markedly enhanced arteriole length density both at 7 days (open bars, n=8) and 14 days (black bars, n=3) compared with saline-injected mice (n=6 and n=4 at 7 and 14 days, respectively). In contrast, CD34− cells did not promote angiogenesis at 7 days (n=3) and induced a modest increase in arteriole length density versus saline-injected mice at 14 days ($P=0.048$, n=3 for both time points). This increase was, however, significantly lower than in mice injected with CD34+ cells at 14 days after injection ($P<0.05$). In vitro–expanded CD34+ cells enhanced arteriole length density at 7 days after injection (n=3). CD34+ cells injected into nonischemic limbs had an angiogenic effect comparable to that observed in ischemic tissues (n=3). Injection of CD34− cells into nonischemic limbs did not increase the formation of new blood vessels (n=3). *$P<0.05$ statistical difference (*t* test). C, Immunohistochemistry on tissue sections from mice adductor muscles injected with CD34+ cells labeled with GFP by retroviral transduction. Several strongly positive GFP+ endothelial cells covering the arteriole lumen are shown. D, Merger of GFP and Hoechst staining of endothelial cells within arterioles in CD34+ cell–injected mice. Arrows indicate GFP+ endothelial cells. Arrowheads indicate GFP− endothelial cells. E, Merger of α-actin (red) and GFP (green) staining in adductor muscles of mice injected with GFP-labeled CD34+ cells. Note the presence of GFP+ endothelial cells covering the lumen of the arteriole and the presence of smooth muscle cells in the arteriole wall (arrowheads). Only sporadically GFP+/α-actin+ cells were observed (arrow), suggestive of CD34+ cell differentiation into VSMCs.

ing that, although possible, CD34+ cells were not preferentially converted into VSMCs.

Angiogenic Potential of Ex Vivo–Expanded CD34+ Cells

A possible limitation to the use of UCB or PB-derived EPCs for therapeutic angiogenesis, is the relatively low number of CD34+ cells that can be recovered from fresh cord blood samples. To overcome this problem, expansion in culture of CD34+-derived endothelial cells has been proposed as a useful strategy to increase the amount of differentiated endothelial cells for blood vessels regeneration.[6] An alternative to this method that was not assessed before is expansion of EPCs in conditions that should preserve their differentiation potency. In fact, several studies have described the use of cocktails of cytokines allowing rapid expansion of HSCs in culture and preserving their engraftment ability in the bone marrow of recipients. Accordingly, UCB CD34+ cells were grown in serum-free medium containing SCF, Flt3 ligand, IL-3, and IL-6 cytokines (adapted from Lazzari et al[27]). In this medium, cell number increased about 10 times in 7 days of culture (not shown) and expression of CD34 antigen was maintained (50.5%±3.16% CD34+ cells after expansion for 7 days). Ex vivo expanded cells maintained an in vivo angiogenic activity to a level comparable to freshly isolated cells at 7 days after ischemia (Figure 3).

Injection of UCB CD34+ Cells Accelerates Muscle Repair In Vivo

Microscopic examination of mouse adductor muscles injected with DiI-labeled CD34+ cells revealed that a number of such cells were not included in newly formed capillaries or arterioles, but were likely localized within muscle fibers (Figure 4A), suggesting that they may be recruited in muscle cell compartment and contribute to muscle repair in ischemic limbs. The results of an immunofluorescence assay on frozen sections of adductor muscles injected with DiI-stained CD34+ cells revealed that some cells expressed desmin (Figures 4B through 4D), a marker for activated satellite cells.[28]

Limb ischemia is a condition characterized by degeneration of vascular and skeletal muscle tissues due to blood flow deprivation (Figure 2B). Hypoxia is one of the major causes of cell death. Regeneration of muscle fibers after ischemia parallels restoration of blood flow by formation of new blood vessels.[29] To assess whether injection of CD34+ cells enhanced muscle regeneration, we determined the density of regenerating muscle fibers in the adductor muscles at 7 days after ischemia. Regenerating muscle fibers can be easily recognized morphologically, due to the presence of nuclei located in the center of the fibers compared with fully developed fibers where the nuclei are located close to the sarcolemma[21,30] (compare Figures 5A and 5B). In tissues treated with freshly isolated CD34+ cells, the density of regenerating fibers was markedly higher compared with controls. Analogous to endothelial differentiation, CD34− did

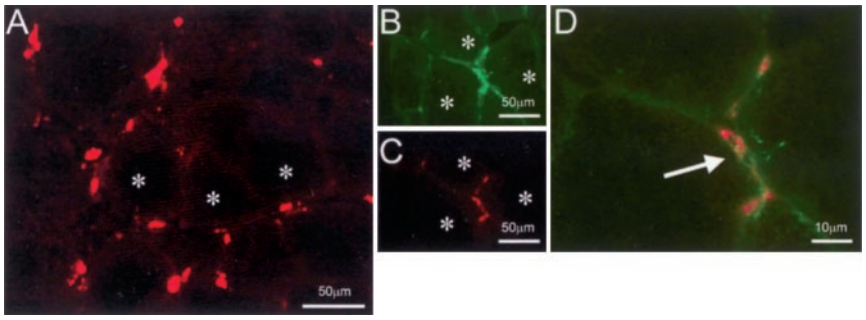

Figure 4. Localization of CD34$^+$-derived cells in the interstitial space between muscle fibers. Frozen sections of DiI-labeled UCB-CD34$^+$–injected adductor muscles. A, DiI-labeled cells localized between muscle fibers (asterisks) were observed. B, Immunohistochemistry for desmin, a marker of activated satellite cells, into two adjacent cells. C, DiI staining in the same cells as in panel B suggests that UCB-CD34$^+$ cells may have converted to a myogenic phenotype. D, Higher magnification of merged panels B and C showing colocalization of desmin and DiI staining (arrow).

not promote myogenic repair, whereas in vitro expanded EPCs improved myogenesis (Figure 5C). Myogenic regeneration in nonischemic muscles injected with either CD34$^+$ or CD34$^-$ cells was also analyzed. Interestingly, as shown in Figure 5C, injection of CD34$^+$ or CD34$^-$ cells into normoperfused muscles did not lead to formation of new muscle fibers. The analysis of muscle regeneration was not extended beyond day 7 because spontaneous regeneration of adductor muscles in ischemic limbs typically peaks between day 14 and 21 after ischemia (not shown), making it impossible to detect a further myogenic effect of CD34$^+$ cells injection.

Direct Participation of CD34$^+$ Cells to Muscle Cell Generation In Vivo and In Vitro

Studies using MyoD-LacZ transgenic mice have shown expression of MyoD in the nuclei of activated muscle satellite cells and the newly formed muscle fibers.[21] Therefore, detection of human MyoD (hMyoD) expression in the nuclei of UCB CD34$^+$-derived cells should allow to test whether injected cells undergo a program of myogenic differentiation. The expression of hMyoD transcription factor in regenerating fibers of mice injected with either CD34$^+$ or CD34$^-$ cells was then studied by immunohistochemistry. For these experiments, we used an antibody that specifically recognizes human but not mouse MyoD (see Materials and Methods). hMyoD staining was found in the nuclei of regenerating muscle fibers (Figure 6A) and in cells that are juxtaposed to the muscle fibers in mice injected with CD34$^+$ cells (Figure 6B). To visualize the position of these cells within the muscle fibers, we performed immunofluorescence on consecutive sections using anti-hMyoD and anti-laminin antibodies (Figures 6F and 6G). By this analysis, we observed that the hMyoD$^+$ cells were localized outside the basal lamina. Interestingly, some regenerating myofibers contained stained and unstained nuclei (Figure 6C), suggesting that CD34$^+$ cells formed mixed human/mouse fibers. Mice injected with CD34$^-$ cells did not show hMyoD staining in regenerating muscle fibers (not shown). To further assess UCB CD34$^+$ cell direct contribution to muscle regeneration, we analyzed sections of adductor muscles of mice injected with GFP-labeled CD34$^+$ cells. As shown in Figure 6E, we found small GFP$^+$ regenerating fibers. These muscle fibers were quantified by counting the number of GFP$^+$ fibers in the total number of regenerating fibers in each section. In a total of 4072 regenerating muscle fibers counted in different sections from 4 mice injected with GFP$^+$ CD34$^+$ cells, we found 232 to be GFP$^+$ (5.6%; Figure 6E).

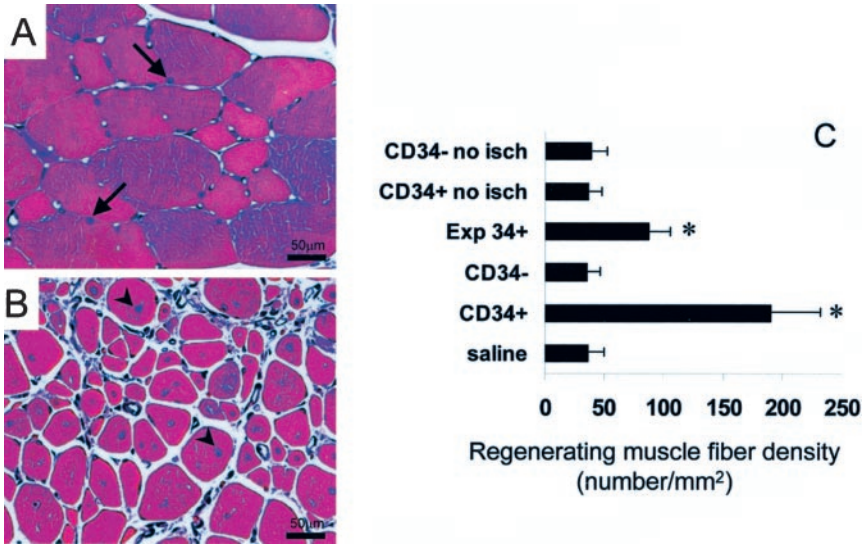

Figure 5. Myogenesis in UCB cell–injected mice. Morphology of adductor muscle and regenerating muscle fiber. A, Control normoperfused tissue showing normal morphology of muscle fibers. Note the presence of several satellite cells with nuclei localized at the periphery of the fibers under the sarcolemma (arrows). B, Regenerating adductor muscle after ischemia in CD34$^+$ cell–injected mice. Note the smaller size of the fibers and the presence of centrally located nuclei (arrowheads), implying fusion of myoblasts. C, Number of regenerating fibers was quantified by counting the total number of fibers showing centrally located nuclei normalized to the section's area. Density of regenerating fibers in each mouse was counted at least in triplicate at different section levels taken at a distance of 250 μm of each other. Graph shows that injection of CD34$^+$ cells caused a significant increase of muscle regeneration compared with CD34$^-$ or saline (CD34$^+$, n=8; CD34$^-$, n=3; saline-injected mice, n=6). Injection of in vitro–expanded CD34$^+$ cells contributed significantly to muscle regeneration analogous to freshly isolated CD34$^+$ cells (n=3). Injection of CD34$^+$ and CD34$^-$ cells into nonischemic muscles (n=3) did not enhance muscle regeneration. *$P<0.05$ (*t* test).

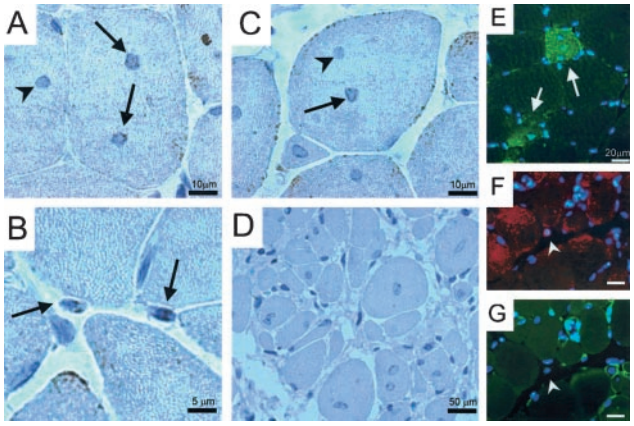

Figure 6. Direct contribution of injected CD34+ cells to muscle regeneration in ischemic mice. A, Regenerating muscle fibers showing human MyoD-stained (arrows) and unstained nuclei (arrowheads). B, hMyoD+ cells were also observed in the interstitial space between the muscle fibers (arrows). It was not possible to determine the position of these cells with respect to the basal lamina of regenerating fibers. These cells may be human CD34+-derived satellite-like cells or CD34+ cells in the process of differentiating into myogenic cells in vivo. C, In some regenerating fibers, it was also noticed the simultaneous presence in the same fiber of two or more nuclei positive (arrow) and negative (arrowheads) for hMyoD staining. This indicates that some regenerating fibers may be composed of mixed human/mouse cells. D, Negative control showing background staining in the absence of hMyoD primary antibody. E, Merger of GFP and Hoechst staining in regenerating muscle fibers obtained by injecting CD34+ cells labeled with GFP. Presence of GFP+-regenerating muscle fibers (arrows) suggests that CD34+ cells contribute to muscle differentiation. F, Immunofluorescence for hMyoD in the adductor muscles injected with CD34+ cells. Arrow indicates a hMyoD+ cell in the interstitial space between muscle fibers. G, Immunofluorescence with anti-laminin antibodies of the adjacent section to that in panel F. Nuclear staining reveals that the hMyoD+ cell in panel F is probably localized outside the basal lamina (green fluorescence surrounding fibers).

Altogether, these results suggest that UCB CD34+ cells have the ability to directly participate to myogenesis in vivo.

In vitro experiments were performed in order to assess the potential of UCB CD34+ cells to differentiate into myogenic cells. According to this hypothesis, DiI-labeled CD34+ and CD34− cells were seeded onto a layer preformed with a line of mouse myoblasts, the C_2C_{12} cells, stable transduced with a retrovirus driving GFP expression. This cell line has been previously reported to sustain myogenic differentiation of muscle-derived side-population (SP) cells.[31] In the presence of 20% FCS (proliferation medium), C_2C_{12} cells proliferate, whereas after starving in 2% horse serum (differentiation medium), C_2C_{12} cells become postmitotic and fuse to form myotubes.[32] During 2 days of culture after seeding, CD34+ cells attached to proliferating myoblasts. Due to coculture condition, it was not possible to determine by quantitative assays whether UCB CD34+ cells proliferated over the layer of cycling myoblasts. However, switching the cultures to differentiation medium caused the appearance of several DiI+/GFP+ myotubes, suggesting myogenic differentiation of UCB CD34+ cells in vitro (Figures 7A and 7B). Because the formation of polynucleate myotubes in culture is a physiological consequence of cell fusion, it is likely that diffusion of both DiI and GFP occurred within myotubes formed by a mixture of DiI-labeled (CD34+) and GFP-labeled (C_2C_{12}) cells (Figures 7C through 7E). A quantification of in vitro myogenic activity was performed by counting the number of DiI+ myotubes obtained by seeding an equal number of CD34+ and CD34− cells onto GFP-labeled myoblasts. As shown in Figure 7F, the number of DiI+ myotubes obtained with CD34+ cells was significantly higher than with CD34− cells, showing that myogenic activity is mostly confined in the CD34+ cell population of human UCB. Recently, it has been found that fusion of neuronal and hematopoietic stem cells to ES cells probably accounts for dedifferentiation.[33,34] For this reason, we assessed the contribution of spontaneous fusion in coculture of DiI+ CD34+ cells onto GFP+ myoblasts before serum starvation. To prevent possible interference by spontaneous myogenic differentiation of C_2C_{12} cells in proliferation medium, we treated feeder cells with 10 Gy ionizing radiation before seeding CD34+ cells. This treatment has been shown to interfere with the normal program

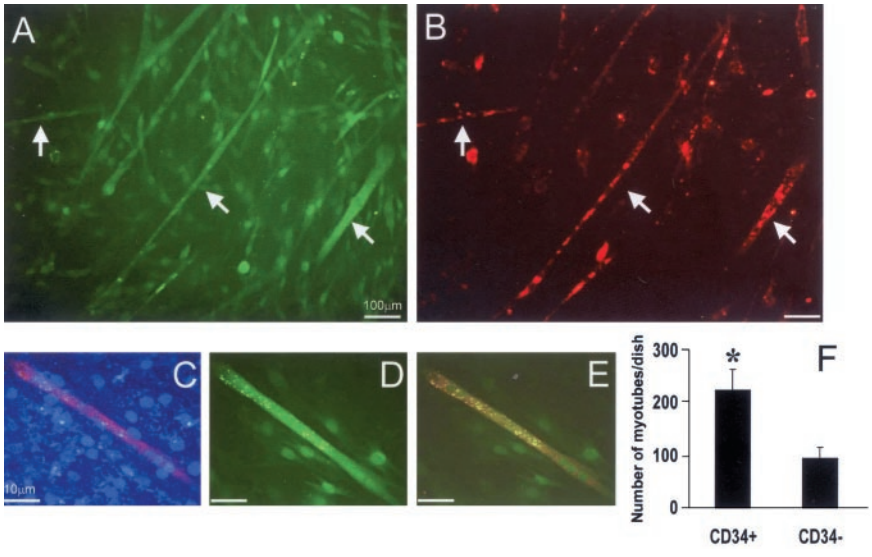

Figure 7. Myogenic differentiation of CD34+ cells in vitro. DiI-labeled CD34+ cells were cocultured onto normal or GFP-expressing C_2C_{12} mouse myoblasts. Myotubes were formed by starvation into 2% horse serum–containing medium (see Materials and Methods). A and B, Low-power view of the same optical field showing GFP (A) and DiI (B) fluorescence of myotubes obtained by coculturing DiI-labeled CD34+ cells onto GFP-labeled C_2C_{12} cells. Note the presence of several GFP+/DiI+ myotubes (arrows). C through E, Hoechst/DiI (C), GFP (D), and GFP/DiI (E) fluorescence of a myotube obtained as a result of fusion of GFP+ myoblasts and CD34+ cells induced by serum starvation. F, Quantification of DiI+ myotubes obtained by reaggregateing DiI-labeled CD34+ and CD34- cells onto GFP-labeled C_2C_{12} cells. Number of myotubes obtained by seeding CD34+ cells was significantly higher than with CD34- cells (n=3). *$P<0.05$ (*t* test).

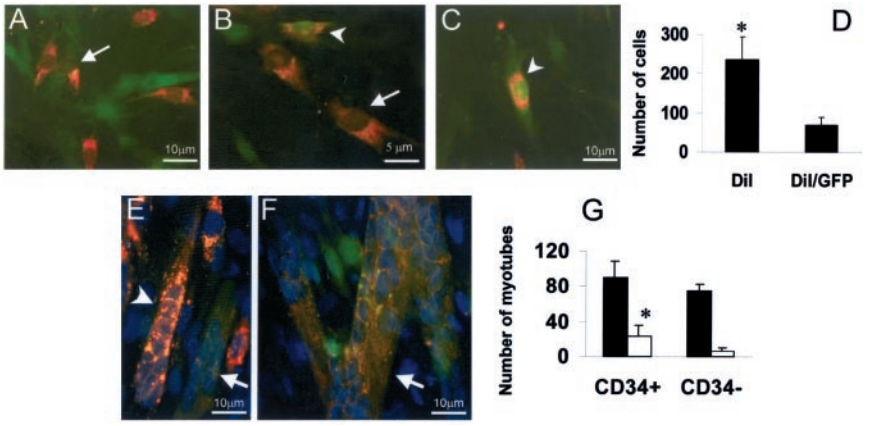

Figure 8. Assessment of cell fusion to myogenic differentiation of $CD34^+$ cells in vitro. To assess whether cell fusion occurs before myogenic differentiation induction, $CD34^+$ cells labeled with DiI were seeded onto X-ray–irradiated GFP^+ C_2C_{12} cells and fixed before induction of myogenic differentiation by serum starvation. A through C, Numerous DiI^+/GFP^- cells were observed (arrows in panels A and B). In some cases, double DiI^+/GFP^+ cells were also found (arrowheads in panels B and C), probably as a result of cell fusion between $CD34^+$ cells and myoblasts. D, Quantification of DiI^+ and DiI^+/GFP^+ cells obtained by coculturing $CD34^+$ cells onto X-ray–treated C_2C_{12} cells (n=3). $^*P<0.05$ (*t* test). E and F, After serum starvation onto normal myoblasts, DiI^+ (arrowheads in panel E), GFP^+ (arrows in panel E), and DiI^+/GFP^+ myotubes (arrows in panel F) were observed, indicative of myogenic differentiation of $CD34^+$-derived cells, C_2C_{12} cells, and mixed $CD34^+/C_2C_{12}$ cells, respectively. G, Quantitative evaluation of DiI^+ (open bars) and DiI^+/GFP^+ (filled bars) myotubes obtained from $CD34^+$ and $CD34^-$ cells after serum starvation (n=4). $^*P<0.05$ (*t* test).

of myoblast myogenic differentiation,[16] thereby allowing discrimination between myoblast and UCB stem cell fusion. In this assay, we found a majority of cells that were stained only with DiI and a significantly lower number of DiI^+/GFP^+ cells (Figures 8A through 8C). We further quantified $CD34^+$ cell-induced fusion by counting the number of DiI^+/GFP^+ versus DiI^+ only cells (Figure 8D). This showed that the number of DiI^+ cells was significantly higher than double DiI/GFP-labeled cells, thus suggesting a relatively low rate of $CD34^+$ cell fusion to C_2C_{12} cells. Unfortunately, due to secondary death of irradiated C_2C_{12} cells, it was not possible to determine whether UCB cells seeded onto irradiated C_2C_{12} cells formed myotubes in differentiation medium. In additional experiments, we thus assessed whether $CD34^+$ cells form myotubes when cocultured onto normal GFP^+ myoblasts by counting the number of DiI^+ myotubes (Figure 8E) and DiI^+/GFP^+ (Figure 8F) myotubes after serum starvation (Figure 8G). Both $CD34^+$ and $CD34^-$ cells gave rise to a number of double-positive myotubes. However, the number of DiI^+ only myotubes obtained by seeding $CD34^+$ was higher than with $CD34^-$ cells. This result, in line with the evidence of a higher myogenic activity in vivo (Figure 5) and in vitro (Figure 7), again suggests that UCB $CD34^+$ cells are capable of myogenic commitment independent of cell fusion.

Discussion

EPCs in the peripheral blood or the umbilical cord blood are a potential source of stem cells from humans to enhance angiogenesis in ischemic tissues. Recent clinical trials of autologous EPC transplantation in patients affected by heart and limb ischemia have recently shown the potential therapeutic benefits of stem cell transplantation to enhance angiogenesis.[9–12] Except for these trials in humans, the potential of hEPCs to generate blood vessels in vivo has been assessed so far in immunodeficient mouse[1,3,6,13] and rat hind limb[4] or heart[8] ischemia models.

UCB $CD34^+$ Cell Injection in Cs-A–Treated Mice Does Not Cause Massive Rejection but Enhances Angiogenesis

The assessment of transplanted human cell rejection was performed by analyzing histological sections of adductor muscles in the proximity of cell injection sites. Except for 2 of 22 cases at day 7 (documented in Figure 2 and the Table), lymphocyte infiltration, necrosis, and tissue fibrosis were comparable to saline-treated mice after ischemia. A relative increase in cell graft failure was noticed at day 14, when about 36% of mice receiving human cells showed signs of generalized tissue inflammation (Table). This suggests that treatment with Cs-A was not completely effective in preventing a certain degree of rejection of human cells or development of GVHD. Because the evaluation of arteriole and muscle fibers density may be altered by tissue inflammation, mice displaying tissue inflammation were excluded from morphometric analyses of blood vessel and/or muscle regeneration (Table). Acute rejection is normally due to the presence of anti-species antibodies in the host. These antibodies mediate organ xenograft failure due to complement activation on endothelial cells of transplanted organs followed by thrombosis. This represents to date one of the major hurdles to the use of interspecies organ transplantation. There are two possible explanations for our finding that only moderate rejection occurred in the ischemic muscles of mice injected with human $CD34^+$ cells. First, transplanted EPCs are not mature endothelial cells and may represent suboptimal targets for the action of preformed anti-species antibody. Second, regenerating blood vessels and muscle fibers likely consisted of mixed host/donor structures (Figures 3 and 6). This may, in part, justify a lower immunogenic potential of neoformed tissues and longer tolerance by the hosts. A discrepancy between our results and previous observations has been found in the different angiogenic response to $CD34^+$ cell injection in nonischemic mice. In fact, it was described that endothelial commitment of EPCs is specifically linked to ischemia[4] or tissue damage.[35] Besides being recruited by angiogenic factors in ischemic tissues, and taking part to

angiogenesis by differentiating into endothelial cells, EPCs per se promote angiogenesis by releasing proangiogenic molecules such as VEGF[15] and angiopoietin1[36] that act on preexisting angioblasts or endothelial cells. One possible explanation for the discrepancies between the present and prior observations[4] is that in the strain of mice used in the present study, responsiveness of host angioblasts and/or endothelial cells to stem cell–produced cytokines is higher than in immunodeficient SCID or inbred mice used previously. Our finding that in a model of immunosuppression in mice the ability of $CD34^+$ cells to differentiate into endothelial cells and to promote the formation of blood vessels in ischemic limbs is maintained represents an advancement toward validation of use of EPCs for allotransplantation in patients.

Myoendothelial Differentiation Pathways of $CD34^+$ Cells: Environmental Control of a Multipotent Cell Type Reprogramming?

Several investigations have shown the existence of different stem cell population in the muscle and the bone marrow that share common markers and may be thus lineage-related. As an example, in mice and humans, CD34 is expressed in circulating and UCB endothelial precursor cells,[1,4,8] but it is also known as a quiescent satellite cell marker in the skeletal muscle and it is expressed in these cells together with myogenic master gene Myf-5.[37] Both skeletal muscle and bone marrow contain a population of cells characterized by low staining by Hoechst 33342 and rhodamine 123 that are thereby called "side population" (SP).[38] Stem cell activity of SP muscle stem cells (MSCs) is not restricted to the myogenic phenotype, as SP cells from the muscle participate to bone marrow reconstitution in lethally irradiated mice.[39] Conversely, bone marrow stem cells are able to differentiate into myogenic cells[39,40] and take part to both cardiac and skeletal muscle regeneration after injury.[41–43] A recent study has described in mice a myoendothelial precursor cell type expressing CD34 in the interstitial space between muscle fibers.[44] These cells have both myogenic and endothelial cell differentiation potency in culture, but in their undifferentiated state, they only express CD34 and *Brcp1* gene product, which is one of the proteins involved in the acquisition of the "side population" phenotype of HSCs.[45] Notably, these cells do not express myogenic markers, but upregulate myogenic master genes such as Pax3 and Pax7 and give rise to mixed myoblast/endothelial colonies in clonal cultures. It is likely that these progenitors represent a myoendothelial stem cell type deriving from either a pool of circulating HSCs or from progenitor cells deriving from cells migrating in the muscle from the dorsal aorta during embryogenesis.[40,44,46] Our results show that an increase in myogenic regeneration by UCB $CD34^+$ cells injection paralleled the increased formation of new arterioles in ischemic tissue. Thus, it is possible that the increase in muscle regeneration was only the consequence of angioblast-mediated recovery of blood flow in the ischemic tissue. As shown in Figures 4 and 6C, however, the presence of $desmin^+$ cells between muscle fibers or the simultaneous presence of nuclei stained with anti-human MyoD protein in regenerating muscle fibers or the occurrence of GFP^+ muscle fibers in mice injected with GFP-labeled $CD34^+$ cells suggests that, at least in part, and similar to a recent mouse model of bone marrow progenitor cell recruitment in the muscle stem cell compartment,[40] $CD34^+$ cells participate to myogenic regeneration after ischemia. In particular, the presence of $hMyoD^+$ cells localized outside the fiber basal lamina (Figure 6B, 6F, and 6G), suggests that a pool of $CD34^+$-derived cells may differentiate into myogenic cells before being recruited into satellite cells pool. These cells, consistently with the observations raised by Tamaki et al[47] in rapidly growing muscles, may be $MyoD^+$ cells derived from injected $CD34^+$ cells that are involved in the formation of new fibers in the interstitial space.

The quantitative evaluation of GFP^+-regenerating muscle fibers (Figure 6) showed that only about 6% of such fibers showed a contribution by injected human cells. This may be explained by the relatively low efficiency in retroviral transduction of $CD34^+$ cells, but also by an underestimation of the effective contribution of $CD34^+$ cells to heal ischemic muscles, due to histological examination of syncytial muscle fibers in transverse sections. In addition, it is possible that only a subset of $CD34^+$ cells is capable of myogenic conversion. Altogether, the results of the present study support the hypothesis that, analogous to the muscle regenerating activity of mesenchymal stem cells[48] or muscle derived stem cells in dystrophic mice (for a review, see Jankowski et al[49]), muscle regeneration after ischemia is induced through a certain degree of direct differentiation of UCB $CD34^+$ cell into myogenic cells.

Trans-Differentiation Rather Than Fusion Accounts for Myogenic Commitment of $CD34^+$ Cells

Figure 7 suggests that $CD34^+$ cells cocultured with mouse myoblasts give rise to muscle cells. This is surprising, as recent work has convincingly shown that mouse bone marrow SP cells cannot be reprogrammed into myogenic cells by coculture onto muscle cells in vitro.[31] One possibility is that UCB angioblasts have a different extent of plasticity compared to mouse bone marrow cells. In fact, it has been reported that human EPCs derived from peripheral blood $CD34^+$ cells[50] and that UCB $CD34^+$ cell fraction contains a higher number of primitive hematopoietic cells compared with human bone marrow.[51] In our analysis, we attempted at deriving human myogenic cells by using conditioned media by both proliferating and differentiating myoblasts (not shown). Thus far, in agreement with results described by Asakura et al,[31] we could not derive any myogenic cells either by using liquid culture or semisolid culture in methylcellulose even in the presence of myoblast conditioned medium. This suggests that cell contact is probably necessary to induce myogenic phenotype in blood borne stem cells. We are currently unaware of whether, analogous to cardiac myocyte conversion of human EPCs[50] or endothelial cells,[52] cell contact is also sufficient to induce myogenic conversion of human EPCs. Work is now in progress to dissect this mechanism by using combinations of adhesion molecules, diffusible factors, and genetic modification of stem cells by viral gene transfer.

Recent results obtained by coculturing somatic stem cells with embryonic stem cells has raised the important issue that spontaneous fusion may account for somatic stem cell dedifferentiation or reprogramming.[33,34] Myogenic differentiation of hematopoietic stem cells, in particular, is potentially subject to interference by fusion since terminal differentiation of muscle fibers in vivo and myotubes in vitro implicates formation of syncytial myotubes as a result of fusion between donor-derived stem cells and myogenic cells in the host. Thus, it may be inferred that formation of regenerating fibers containing donor-derived nuclei stained for a myogenic marker such as MyoD or even appearance of GFP^+ fibers (Figure 6) may result from fusion rather than to a real reprogramming event. On the other hand, we surmise that spontaneous fusion does not entirely account for the process of $CD34^+$ cell-mediated muscle repair as we found that (1) increase of myogenesis only occurred by injecting ischemic mice with $CD34^+$ cells (Figure 5), (2) formation of myotubes in culture was higher by culturing $CD34^+$ cells onto myoblasts compared with $CD34^-$ cells (Figure 7F), (3) the majority of $CD34^+$-derived cells in coculture were not fused to myoblasts before induction of myogenic differentiation by serum deprivation (Figures 8A through 8D), and (4) DiI^+ only myotubes were more frequently scored by seeding $CD34^+$, rather than $CD34^-$ cells, onto GFP-labeled C_2C_{12} myoblasts (Figure 8G). In addition, generation of new muscle fibers only occurred in tissues damaged by ischemia (Figure 5). Taken together these findings suggest that determination of myogenic phenotype is specific for $CD34^+$ EPCs and that fusion with preexisting myogenic cells in vivo (Figure 6) occurs preferentially under stimulation by physiological signals that are upregulated on tissue damage, or in vitro (Figure 7) following a differentiative trigger such as serum deprivation.

Conclusion

The existence of peripheral blood/bone marrow stem cell plasticity is currently hotly debated. Numerous studies have provided both positive and negative demonstrations of bone marrow stem cell conversion into skeletal and cardiac myocytes, and its potential clinical relevance.[31,35,39,41,53,54] It has been hypothesized that tissue damage is one of the conditions that might be involved in stem cell lineage barrier transition and *trans*-differentiation.[55] Our findings, showing that human $CD34^+$ cells may induce angiogenesis also in nonischemic mice, suggest that an angioblast "default" differentiation pathway into endothelial cells is activated even without damage-associated stimuli. By contrast, induction of myogenic phenotype in $CD34^+$ cells appears to be exclusively linked to the presence of ischemic condition in host tissue. This observation is in line with evidences showing that contribution of recruited hematopoietic stem cells to skeletal and cardiac muscle repair is strongly enhanced by tissue injury.[40,56] At present, the identity of factors that commit $CD34^+$ stem cells toward endothelial and/or myogenic phenotype in ischemic tissues is controversial. However, an array of cytokines and chemokines (VEGF, PlGF, SDF-1, GM-CSF, SCF, and HGF) that are involved in mobilization of EPCs in the peripheral blood after ischemia or after plasmid or viral vector-mediated gene transfer have been identified.[57–60] It is still a matter of speculation whether multiple signaling by these factors or an interplay between these factors and intracellular pathways activated by adhesion molecules such as integrins, inflammatory cytokines, or inducing signals, eg, Wnt signaling, play a role in ischemia-induced recruitment and, possibly, *trans*-differentiation of stem cells. Future studies addressing this question are therefore necessary to unravel how stem cell–mediated regeneration may be triggered to heal ischemic tissues.

Acknowledgments

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