Postnatal neovascularization by endothelial progenitor cells immortalized with the simian virus 40T antigen gene

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Abstract. Endothelial progenitor cells (EPCs) contribute to blood vessel formation in ischemic and tumorous tissues, but comprise only a small population in circulation. We attempted to immortalize putative EPCs from human cord blood. Human CD34$^+$ cord blood cells were cultured in the presence of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF), and transfected with a retroviral vector encoding the simian virus 40 large T (SV40T) antigen. This resulted in the immortalization of cord blood cells, leading to the establishment of several cell lines. One of these lines, HYCEC-1, exhibited a phenotype characteristic of the endothelial lineage, including expression of von Willebrand factor and VEGF receptor-2 (VEGFR-2/KDR/Flk-1) and uptake of acetylated-low density lipoprotein. Flow cytometric analysis revealed that HYCEC-1 cells were strongly positive for CD31 and CD146, moderately positive for CD144, weakly positive for CD133 and CD34, and negative for CD14 and CD45. HYCEC-1 cells formed capillary-like structures on basement matrix gel *in vitro*. Upon transplantation into the ischemic hind limb of nude rats, HYCEC-1 cells efficiently participated in neovascularization and augmented blood flow. The immortalized HYCEC-1 cells are suggested to be a class of EPCs that can efficiently participate in postnatal neovasculogenesis in the ischemic hind limb, and may also be a useful tool for studying tumor vessel formation.

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Introduction

Endothelial progenitor cells (EPCs), which differentiate into mature endothelial cells (ECs) that line the lumen of blood vessels, are mobilized from the bone marrow into the peripheral blood and contribute to the vasculogenesis of ischemic tissues (1-4). Increasing evidence has indicated that tumor blood vessel formation is supported by bone marrow-derived precursor cells, including EPCs (5). Human umbilical cord blood is also known to be a rich source of EPCs (6,7). Circulating EPCs are characterized by the expression of CD34$^+$ and vascular endothelial growth factor receptor-2 (VEGFR-2/KDR/Flk-1) (1-4). Transplantation of EPCs into the ischemic limb or heart results in the augmentation of neovascularization and reduction of the ischemic state (1-4,6). However, the absolute number of EPCs in the bone marrow, peripheral blood or cord blood is low (1,4,6). This low number, together with the difficulty of expanding EPCs *ex vivo* due to their limited life span, has hampered clinical applications of EPCs.

The immortalization of EPCs might enable highly efficient *ex vivo* expansion of EPCs and allow the preparation of an optimal number of EPCs for transplantation. Transfection of EPCs with the human telomerase reverse transcriptase (hTERT) gene resulted in increasing EPC potency, but not in immortalization (8). Several human endothelial cell lines have been established using microvascular and umbilical vein endothelial cells (9-12). However, these cells are mature endothelial cells (EC) and do not have the characteristics of EPCs. In this study, we attempted to immortalize EPCs by transfecting simian virus 40T (SV40T) into CD34$^+$ cord blood cells cultured under angiogenic conditions (12-15). CD34$^+$ cells differentiate into spindle-shaped adherent EPCs in the presence of VEGF and basic fibroblast growth factor (b-FGF) (1,6), accompanied by the acquisition of endothelial markers (16). Here, we show that one of the cell lines established, HYCEC-1, exhibits characteristics of partially differentiated EPCs with the capacity to generate vessel-like structures *in vitro* and efficiently participate in neovasculo-

genesis *in vivo*. Since HYCEC-1 can expand efficiently, resulting in the generation of a large number of EPCs, this cell line may be of use for exploring the potential of EPC therapy, and studying the role of EPCs in neovascularization of ischemic tissues and tumor tissues.

Materials and methods

Cell preparation. Umbilical cord blood (CB) was obtained from normal full-term deliveries with the mothers' consent. Mononuclear cells (MNC) were separated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). $CD34^+$ cells were separated from MNC using a Direct CD34 Progenitor Cell Isolation kit and the automated magnetic cell sorter system (autoMACS) (Miltenyi Biotec, CA). The purity of $CD34^+$ cells was >92% (Fig. 1B). Human umbilical blood endothelial cells (HUVEC) were isolated and cultured as described by Jaffe *et al* (17).

Culture of $CD34^+$ CB cells under angiogenic conditions. $CD34^+$ cells ($2\text{-}5x10^5$) were cultured in 60 mm dishes coated with collagen type I (Becton-Dickinson, Mountain View, CA, USA) containing 2 ml of α-MEM supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), 10% horse serum (Gibco), 2 mM glutamine, 10 μg/ml heparin (Sigma Chemical Co., St. Louis, MO), 20 ng/ml VEGF and 5 ng/ml basic fibroblast growth factor (b-FGF) (R&D Systems, Minneapolis, MN) at 37˚C in humidified air with 5% CO_2.

Production of recombinant retroviral vector SSR#69. The retroviral vector, SSR#69, which carries the SV40T immortalizing gene, hygromycin-resistant gene (HygroR) and Herpes simplex virus-thymidine kinase (HSV-TK) gene, which is flanked by loxP recombination targets and removable by Cre recombinase (Fig. 1A), was produced as described previously (12-15). The Amphotrophic Ψ Crip packaging cells producing SSR#69 were cultured in a T75 flask (Falcon) at 32˚C in 5% CO_2, and the supernatant containing the retroviruses was cryopreserved in an aliquot at -80˚C until use (14).

Transfection of $CD34^+$ cells with SV40 T antigen. $CD34^+$ cells, cultured under angiogenic conditions as described above starting on day 3 of culture, were transduced 6 times with the Ψ Crip supernatant every 2 days, then subjected to hygromycin B selection 2 days later. One of the hygromycin-resistant colonies with a particularly high proliferative capacity was termed HYCEC-1 and maintained in 60 mm dishes.

RT-PCR. Total RNA was extracted using an Isogen RNA Extraction kit (Wako Pure Chemical Co., Osaka, Japan) and dissolved in water treated with diethyl pyrocarbonate (DEPC) (Sigma). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the RNA PCR kit AMV ver. 2.1 (Takara Shuzo, Shiga, Japan). The amplified products were electrophoresed in 2% agarose gels (Sigma) and stained with ethidium bromide. The primers were: SV40T, sense CAGGCATAGAGTGTCTGC, and antisense CAACAGCCTGTTGGCATATG (product 422 bp); Flk-1, sense CTGGCATGGTCTTCTGTGAAGCA, and antisense AATACCAGTGGATGTGATGCGG (product 790 bp), and ß-actin, sense TGCTATCCAGGCTGTGCTAT, and antisense GATGGAGTTGAAGGTAGTTT (product 446 bp).

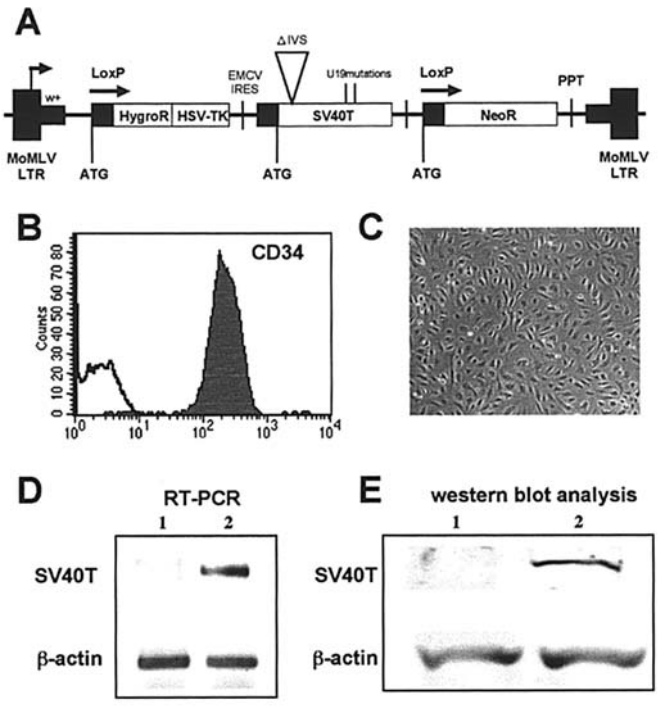

Figure 1. (A) Schematic drawings of the retroviral vector SSR#69. The genes of HygroR/HSV-TK and SV40Tag are expressed in the transduced cells with SSR#69. (B) Expression of CD34 on cord blood cells. $CD34^+$ cord blood cells were immunomagnetically separated and stained with a PE-labeled CD34 antibody that reacts with different epitopes of the CD34 antigen, then analyzed by flow cytometry. (C) Morphology of HYCEC-1 at confluency (10 passages). Phase contrast microphotographs at a x100 magnification. (D) SV40T mRNA (422 bp) was detected by RT-PCR analysis in attached cells (lane 1) and HYCEC-1 cells (lane 2). ß-actin (446 bp) was used as a control. (E) Western blot analysis of SV40T antigen (94 kDa). Actin (43 kDa) was used as a control. LTR, long terminal repeat; SV40T, simian virus 40 T antigen; HSV-TK, Herpes simplex virus thymidine kinase; HygroR, hygromycin-resistant gene; NeoR, neomycin-resistant gene.

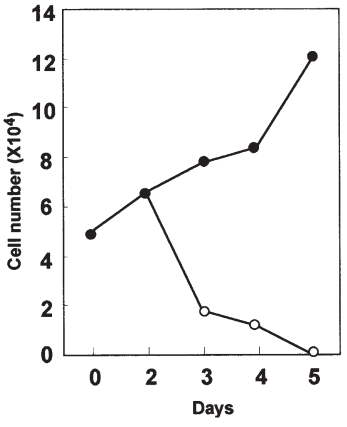

Figure 2. Growth curve of HYCEC-1 cells in the absence or presence of ganciclovir. Results are representative data of three separate experiments. HYCEC-1 cells ($5x10^4$) were cultured with (open circle) and without (closed circle) 5 μM ganciclovir, and viable cells were counted after Trypan blue staining.

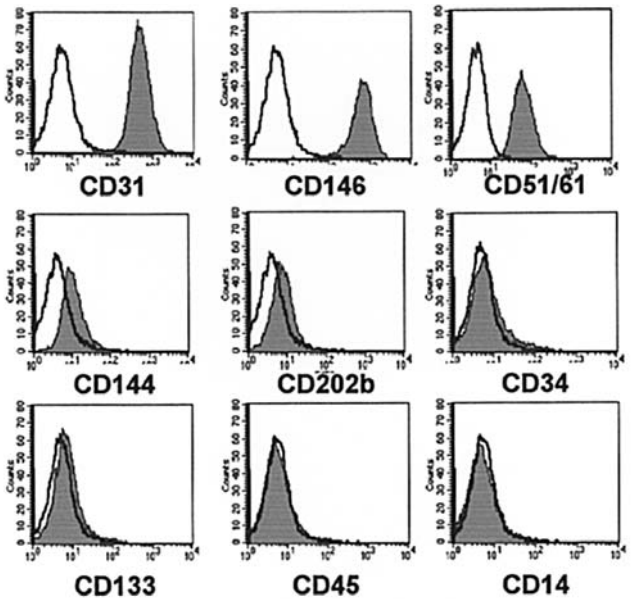

Figure 3. Surface marker analysis of HYCEC-1 cells. Cells were incubated with FITC- or PE-conjugated antibodies and analyzed by flow cytometry.

Flow cytometric analysis. Cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) at 4˚C for 30 min, and subjected to immunofluorescence analysis using an Epics XL flow cytometer (Coulter, Miami, FL). The monoclonal antibodies used were: FITC-labeled mAbs for CD29 (Coulter), CD31, CD44, CD49d, CD54, and CD62L (Immunotech, Marseille, France), CD105 (Serotec, Oxford, UK), and PE-labeled CD34 (Immunotech) and CD106 (Pharmingen, San Diego, CA). Non-labeled mAbs to vascular endothelial (VE)-cadherin (CD144), endothelial cells (clone P1H12, CD146) (Chemicon International Inc., Temecula, CA) and Tie-2/TEK (CD202b) (Nichrei Corporation, Tokyo, Japan) were also used as primary Abs with proper FITC- or PE-labeled secondary Abs. In some experiments, endothelial cells were stimulated by 10 ng/ml of TNF-α (R&D Systems) for 24 h prior to flow cytometric analysis. Dead cells were gated out with a forward versus side-scatter window and propidium iodide staining. The appropriate isotype-matched control antibodies were used in all experiments.

Immunofluorescence assay. HYCEC-1 cells were cultured on a Chamber slide (Becton-Dickinson), fixed with cold methanol, incubated with anti-vWF mAb (Dako A/S, Denmark) and then with polyAb anti-mouse IgG (H+L chain)-FITC (MBL), and examined under a fluorescence microscope.

Cellular uptake of acetylated LDL. HYCEC-1 cells were cultured on a Chamber slide in the presence of 10 μg/ml Dil-labeled acetylated-LDL (Dil-Ac-LDL; Molecular Probes, Eugene, OR, USA) for 4 h at 37˚C. The cells were then examined for uptake of the fluorescent dye Dil under a fluorescence microscope using a rhodamine filter.

Western blot analysis. Western blot analysis was performed as previously described (18). Briefly, the cells were lysed with buffer containing NP-40, electrophoresed in a 10% SDS-polyacrylamide gel (Dai-ichi Kagaku, Tokyo, Japan), and blotted onto a Hybond P membrane (Amersham). The membrane was incubated with anti-SV40 antibody (mouse IgG; Santa Cruz), then with alkaline phosphatase-conjugated goat anti-mouse IgG (Santa Cruz). The membrane was treated with nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (Sigma) to visualize the protein bands.

In vitro angiogenesis model. HYCEC-1 cells grown in collagen-coated culture dishes were harvested by treatment with 0.05% trypsin/0.25 mM EDTA, cultured on basement membrane matrix gels (Matrigel; Becton-Dickinson) for 24 h, and examined for the formation of endothelial cell networks under a microscope.

In vivo angiogenesis model. A rat model of surgically induced hind limb ischemia was employed using immunodeficient nude rats (F344/N rnu/rnu) (Japan Clea, Tokyo). To create a hind limb ischemia model, the left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg). The distal portion of the saphenous artery and all side branches and veins were dissected and excised. The right hind limb was kept intact and used as the non-ischemic limb. The following day, HYCEC-1 cells ($3x10^5$ cells per rat) were injected into the ischemic thigh muscle at five different points with a 26-gauge needle. After 3 weeks, the rats were sacrificed, and ischemic skeletal muscles were isolated. Immunohistochemical staining was performed using a human-specific anti-CD31 antibody that does not crossreact with rat CD31 (clone JC/70A; Dako Japan, Kyoto). A Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) were used to visualize positive reactions. Histochemical staining for alkaline phosphatase was also used to detect capillary endothelial cells in both ischemic and non-ischemic skeletal muscle tissues as described (6).

Laser Doppler perfusion image of hind limb blood flow. We measured the ratio of ischemic (left) and normal (right) limb blood flow in terms of the LDPI index using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK). Changes in the frequency of laser Doppler were displayed as a color-coded image representing blood flow. High flows were displayed in a red to white color, and low to no flows in a dark blue color. The stored images were subjected to computer-assisted quantification of blood flow to obtain the average flow.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). The Student's t-test was used to compare two groups, and p-values <0.05 were regarded as statistically significant.

Results

Immortalization of cord blood cells. Human $CD34^+$ cord blood cells (Fig. 1B) were cultured on collagen type-I-coated dishes in the presence of VEGF and b-FGF, with half of the medium changed every 3 days to discard floating cells. Starting on day 3 of culture, we infected these cells with SSR#69 virus 6 times every 2 days, and selected for hygromycin resistance (Fig. 1A). Of 20 colonies, 3 grew, and one with a good

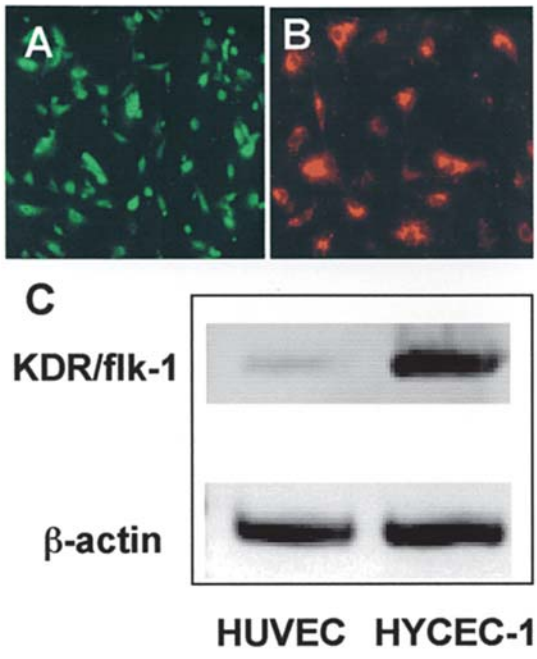

Figure 4. (A) Expression of vWF in HYCEC-1 cells. HYCEC-1 cells were fixed on Chamber slides, incubated with anti-vWF mAb and an FITC-labeled second antibody, and examined under a fluorescence microscope. (B) Uptake of Ac-LDL by HYCEC-1 cells. HYCEC-1 cells were cultured on Chamber slides in the presence of Dil-labeled acetylated-LDL for 4 h at 37˚C. The cells were examined for uptake of Dil under a fluorescence microscope using a rhodamine filter. (C) RT-PCR analysis of the expression of KDR/flk1 (790 bp) mRNA in HUVEC and HYCEC-1 cells. ß-actin (446 bp) was used as an internal control.

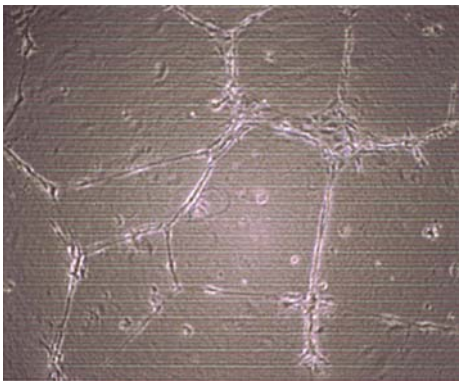

Figure 5. The HYCEC-1 cells formed angiogenic webs on basement membrane matrix gel. Phase contrast microphotograph at an original magnification of x100.

proliferative capacity in the presence of VEGF and b-FGF was named HYCEC-1 and characterized in depth. HYCEC-1 cells were observed to be spindle-shaped at low concentrations, and cobblestone-like and contact-inhibited at confluency (Fig. 1C). HYCEC-1 cells express SV40T mRNA (Fig. 1D) and protein (Fig. 1E), but show no tumorgenicity following subcutaneous injection into nude rats for at least 4 months (data not shown). The doubling time of HYCEC-1 cells was determined to be approximately 3 days (Fig. 2). Since HYCEC-1 cells carry the HSV-TK gene, the addition of ganciclovir to the culture medium results in cell death (Fig. 2).

Endothelial nature of HYCEC-1 cells. Flow cytometric analysis revealed that HYCEC-1 cells express several endothelial-

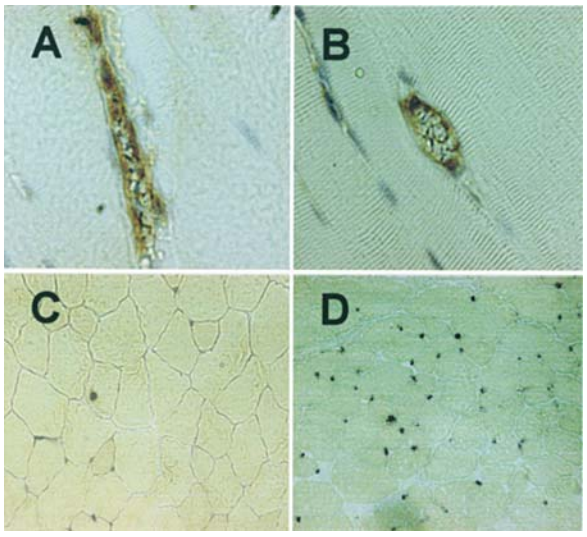

Figure 6. Participation of HYCEC-1 cells in neovascularization *in vivo*. (A and B) Tissues were harvested from the ischemic hind limb of a nude rat 3 weeks after transplantation of HYCEC-1 cells, and stained with anti-human CD31 antibody. (C and D) Alkaline phosphatase staining for the detection of capillary density from the ischemic hind limb of a nude rat. (C) Untreated and (D) treated with HYCEC-1.

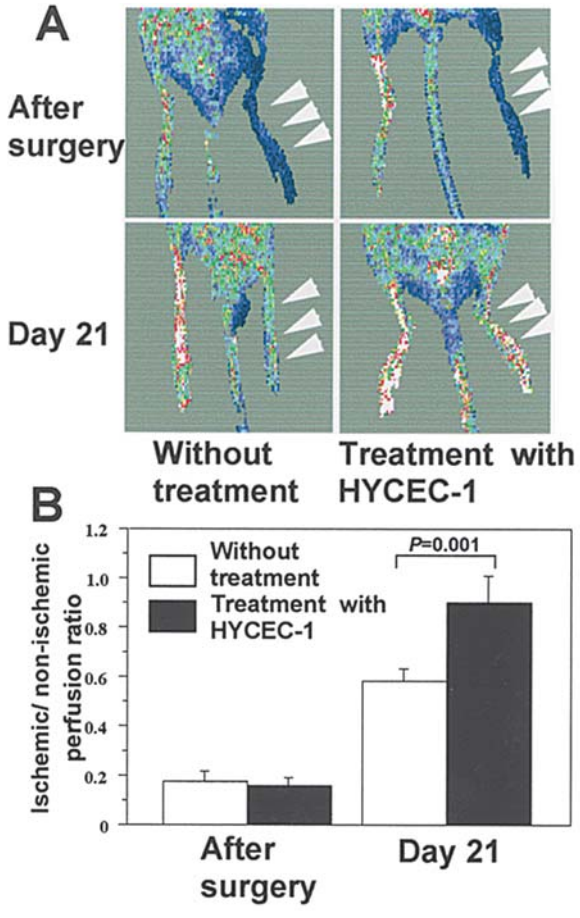

Figure 7. Augmented neovascularization by local transplantation of HYCEC-1 cells. (A) Representative results of LDPI analysis of the left hind limb (arrowhead) 1 day after left femoral artery ligation and 21 days later. Low to no flow is shown in the dark blue color, and high flow is shown in the red to white color. (B) Quantitative analysis of perfusion recovery measured by LDPI in untreated (n=6) and treated (n=6) groups with HYCEC-1 cells. The LDPI index was expressed as the ratio of left (ischemic) and right (non-ischemic) limb blood flow.

specific markers: CD31 (PECAM-1) and CD146 (P1H12) were expressed strongly, CD144 (VE-cadherin) and CD202b (TEK) moderately, and CD34 and CD133 weakly (Fig. 3). CD14 and CD45 were not expressed (Fig. 3). In addition, potent expression of CD29 (VLA-ß), CD44 (HCAM) and CD105 (endoglin), and weak expression of CD49d (VLA-α) were observed (data not shown). CD54 (ICAM-1) was expressed weakly, but its expression was up-regulated markedly upon stimulation with TNF-α (data not shown). Immunofluorescence assays showed that HYCEC-1 cells expressed vWF strongly (Fig. 4A) and uptook Dil-Ac-LDL (Fig. 4B). The expression of KDR/Flk-1 mRNA in HYCEC-1 cells was found to be much higher than that in HUVEC (Fig. 4C).

HYCEC-1 cells exhibit the ability to participate in angiogenesis in vitro. Cultivation of HYCEC-1 cells on basement matrix gel for 24 h resulted in the formation of branched tube-like structures (Fig. 5), indicating that HYCEC-1 cells had the capacity to participate in angiogenesis *in vitro*.

HYCEC-1 cells participate in neovascularization in nude rats. We examined whether transplanted HYCEC-1 cells were able to participate in postnatal neovascularization *in vivo*. We ligated the left femoral artery of nude rats to induce unilateral hind limb ischemia, transplanted HYCEC-1 ($3x10^5$ cells/rat) into the ischemic thigh skeletal muscle, and immunohistochemically analyzed human-specific CD31-positive endothelial cells in the thigh muscle 21 days later. We found CD31-positive cells in capillary structures in which red blood cells were identified in the lumen (Fig. 6A and B). As the human-specific anti-CD31 antibody did not crossreact with rat CD31, these results indicate that the locally injected HYCEC-1 cells differentiated into mature EC and formed blood vessels. Histochemical staining for alkaline phosphatase in the ischemic muscles also revealed a significant increase in capillary density in the rats transplanted with HYCEC-1 cells (Fig. 6C and D). To quantify the contribution of HYCEC-1 cells to postnatal neovascularization in the ischemic hind limb, Laser Doppler perfusion image analysis of blood flow was performed (Fig. 7). We found increased blood flow in the left hind limb transplanted with HYCEC-1 cells at postoperative day 21 (Fig. 7A). The LDPI index (ratio of ischemic/normal hind limb blood flow) of the HYCEC-1-transplanted group was significantly higher than that of the control group (P=0.001) (Fig. 7B). Thus, local transplantation of HYCEC-1 cells augmented blood flow in the ischemic hind limbs of nude rats.

Discussion

In the present study, human EPCs were successfully immortalized by culturing $CD34^+$ cord blood cells under angiogenic conditions, then transfecting them with a retroviral vector encoding SV40 large T antigen. It has been shown that transfection with the human telomerase reverse transcriptase (hTERT) gene results in the enhancement of angiogenic properties of EPCs without immortalization (8). HYCEC-1 was established here as a human cell line with the functional characteristics of human EPC.

HYCEC-1 cells are spindle-shaped and require VEGF and b-FGF for proliferation. They express vWF and incorporate acetylated LDL, indicating that HYCEC-1 is of endothelial cell lineage (Fig. 4). Although HYCEC-1 cells are derived from $CD34^+$ cord blood cells, they only weakly express the hematopoietic/endothelial stem cell markers, CD34 and CD133. Instead, HYCEC-1 strongly expresses the mature endothelial cell markers CD31 (PECAM-1) and CD146 (P1H12), and moderately expresses the immature endothelial markers CD144 (VE-cadherin) and CD202b (TEK). HYCEC-1 produces much higher levels of KDR/Flk-1 mRNA than the mature ECs, HUVEC (Fig. 4). Although CD146 is often used as a marker of mature ECs, endothelial precursor cells cultured from $CD34^+$ bone marrow cells also express CD146 (19). A precise marker of EPCs has yet to be clearly defined because of the considerable overlap between EPCs and mature ECs (4). Furthermore, there seems to be a hierarchy of EPCs, accompanied by the loss of hematopoietic markers, acquisition of endothelial markers during angiogenic differentiation (16), and early and late EPC subtypes with respect to maturation (20). We consider HYCEC-1 cells to be an intermediary between early progenitor cells and fully mature ECs.

HYCEC-1 form a network on matrix gel in culture in the absence of HUVEC (Fig. 5), although immature cord blood EPCs require the presence of HUVEC for network formation (6). Transplanted HYCEC-1 efficiently participated in postnatal neovascularization and full lumen formation (Fig. 6A and B), and also augmented regional blood flow *in vivo* (Fig. 7), indicating that HYCEC-1 is a functional EPC line. Taken together in terms of both phenotypical and functional aspects, HYCEC-1 is suggested to be a partially differentiated EPC line with an extensive capacity for neovascularization.

Local injection of autologous bone marrow mononuclear cells in ischemic limbs has been shown to reduce ischemic symptoms and augment neovascularization in humans (21). It has not been elucidated as to whether the directly injected bone marrow EPCs participate in neovascularization, or if angiogenic factors secreted from the bone marrow cells are responsible for blood vessel formation. A previous study suggested that human EPCs prepared from the peripheral blood are derived from monocytes/macrophages and certain secreted angiogenic factors likely mediate the angiogenic effect (22). However, the present results suggest that clonally expanded EPCs are able to efficiently and directly participate in neovascularization.

In the mouse embryonic stem cell system, Flk-1-positive cells serve as vascular progenitor cells (VPCs) (23). Immature $Flk\text{-}1^+$/VE-cadherin $(CD144)^-$/PECAM-1 $(CD31)^-$ VPCs do not participate in neovasculogenesis, while mature $Flk\text{-}1^+$/VE-cadherin $(CD144)^+$/PECAM-1$(CD31)^+$ VPCs, obtained by culturing immature VPCs with VEGF for 3 days, do efficiently participate (24). Thus, endothelial progenitors at more differentiated stages may be more effective in enhancing neovasculogenesis than immature cells. Consistent with this hypothesis, the findings presented here show that HYCEC-1 is a cell line of partially differentiated human EPCs with the ability to efficiently participate in vasculogenesis. These results suggest that the identification and isolation of appropriately differentiated human EPCs may be a crucial development in the search for a more effective EPC therapy.

Cord blood has been increasingly used for hematopoietic stem cell (HSC) transplantation since it contains a high concentration of HSCs and is available via a potentially large number of donors without invasive procedure. Considering the fact that cord blood is a much more robust source for EPCs than adult peripheral blood (6), cord blood could be a vital alternative source of EPCs in the future.

Although SV40 large T antigen has been used to immortalize a variety of human cell types, it is possible that SV40 large T antigen is oncogenic in humans (25). We observed no development of tumors in rats injected subcutaneously with HYCEC-1 cells for at least 4 months. Furthermore, the system we used carries certain important safeguards against this risk: i) incorporation of a 'suicide' gene (HSV-TK) that eliminates cells carrying the SV40 T gene in the presence of ganciclovir (Fig. 2); and ii) elimination of the SV40 T gene by site-specific recombination (13). Thus, we pursued a relatively 'safe' procedure for applying this approach to any desired experimental and clinical application. On the other hand, the involvement of EPCs in tumor vessel formation has been reported, mainly in rodents (5). HYCEC-1 may also provide a means for clarifying the contribution of human EPCs to tumor vessel formation, i.e. by using a xenotransplantation model system.

In summary, we obtained the cell line HYCEC-1 from $CD34^+$ cord blood cells, and demonstrated that this cell line is able to expand clonally. These cells exhibit characteristics of relatively differentiated EPCs and are able to efficiently participate in neovascularization in ischemic hind limbs on cell transplantation. Thus, the HYCEC-1 cell line may facilitate research into the mechanisms of postnatal neovasculogenesis in both ischemic and tumorous tissues.

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