Defining Report

Identification of the Adult Human Hemangioblast

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ABSTRACT

Recent studies show that human CD133⁺ (previously known as AC133⁺) cells from mobilized peripheral blood consist of stem cells with either hematopoietic or endothelial potential. To test whether this population also contains individual precursors with both capacities, the defining characteristics of the elusive adult hemangioblast, we developed a culture system that allows single-cell analyses of differentiation. In the presence of vascular endothelial growth factor (VEGF), stem cell growth factor (SCGF), and FLT-3 ligand, CD133⁺-enriched cells were first expanded and the amplified cells were transduced with a vector encoding an enhanced green fluorescent protein (EGFP) marker gene. Single EGFP⁺ cells were then cocultured with corresponding nontransduced cells from the same experiment, yielding 50–100 marked cells in 8% of the wells after 2 weeks. The resultant cells were divided and differentiated with either granulocyte colony-stimulating factor (G-CSF) or with SCGF and VEGF. These culture conditions resulted in the formation of neutrophil or endothelial cells, respectively, as identified morphologically and by phenotypic staining. Dual differentiation of EGFP⁺ cells could be observed in one-quarter of clones from single-seeded cells, suggesting that 2% of EGFP⁺ cells were in fact human hemangioblasts. These cells could be expanded for at least 28 days without losing this dual capacity. Hence, this culture system may be of clinical relevance in the development of cellular therapies for disorders involving hematopoiesis and the vascular system. In addition, our results provide important information related to the development of the vasculature and the potential role of hemangioblasts in vasculogenesis in adult humans.

INTRODUCTION

AGROWING BODY OF EVIDENCE suggests a close developmental relation between hematopoiesis and vasculogenesis in vertebrates. During early embryogenesis, hematopoietic and endothelial lineages derive from aggregates of mesodermal cells that subsequently mature and form blood islands in the extraembryonic yolk sac. These blood islands consist of an inner core of blood cells and external layer of endothelial cells (1,2). The simultaneous emergence of the hematopoietic and endothelial lineages within the blood islands has led to the hypothesis

that they originate from a common precursor referred to as the hemangioblast (2). The concept of a common ancestral cell has been supported by the observation that the two lineages possess several phenotypic and molecular similarities (3–8). In addition, gene targeting experiments have shown that mutations of single mouse or zebrafish genes result in defective development of both lineages (9–13). Studies on zebrafish embryos demonstrating that ectopic expression of the transcription factor SCL in early mesoderm results in an overproduction of hemangioblasts provide further evidence for its existence (14). Recently, the in vitro equivalent of the hemangioblast has been iden-

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MATERIALS AND METHODS

Input cells

Cells were isolated from frozen leukapheresis products of 9 patients undergoing high-dose chemotherapy (HDC) with autologous hematopoietic progenitor cell (HPC) support. Peripheral blood (PB) progenitor cells were collected by leukapheresis after mobilization of HPC with recombinant human granulocyte colony-stimulating factor (G-CSF), administered subcutaneously (s.c.) at 5 $\mu\text{g/kg/day}$ for up to 7 days.

Enrichment of CD133⁺ cells

After thawing, products were resuspended in phosphate-buffered saline (PBS; Life Technologies, Karlsruhe, Germany) supplemented with 0.5% human serum albumin (HSA; Blood Donating Service of the German Red Cross, Hamburg, Germany) and 0.6% ACD-A. Deoxyribonuclease I (DNase I; Sigma, Deisenhofen, Germany) was added at 100 U/ml, and the specimens were shaken gently for 30 min at room temperature. Mononuclear cells (MNC) were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) for 20 min at $500 \times g$ and washed two times in PBS at $300 \times g$ to remove platelets. MNCs were then resuspended in PBS, incubated with CD133 monoclonal antibody (mAb)-conjugated paramagnetic microbeads (CD133 Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany), washed, and then loaded on an automatic magnetic separation column device (AutoMACS, Miltenyi Biotec). CD133⁺ cells were isolated using the software program Posseldx (Miltenyi Biotec). For all preparative steps, as well as for the separation procedure, PBS was supplemented with 0.5% HSA and 0.6% ACD-A. The purity and phenotype of the CD133⁺ cells was determined by flow cytometry.

Cultures of human CD133⁺ cells

Freshly isolated CD133⁺ cells were cultured in fibronectin-coated 24-well plates (fibronectin from Life Technologies, plates from Greiner, Hamburg, Germany) at a density of 2×10^6 cells/ml in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Sigma), 10% horse serum (Sigma), and 10^{-6} M hydrocortisone (Sigma). In pilot studies, various growth factor combinations were compared for their ability to induce proliferation, including vascular endothelial growth factor (VEGF; 50 ng/ml), basic fibroblast growth factor (bFGF; 10 ng/ml), hepatocyte growth factor (HGF; 10 ng/ml), stem cell growth factor (SCGF; 100 ng/ml), stem cell factor (SCF; 100 ng/ml), FLT-3 ligand (50 ng/ml), epidermal growth factor (EGF; 10 ng/ml), in-

tified (15–17) and characterized as a transient cell during embryonic development (16).

The emergence of the blood islands in the yolk sac marks the onset of vascularization in the developing embryo. Two different processes contribute to the formation of the vascular system. The first process, vasculogenesis, requires the differentiation of endothelial cells from hemangioblasts and their subsequent organization into a primary capillary plexus (18,19). The second process, angiogenesis, results in the formation of new vessels by sprouting from preexisting blood vessels (20,21). Primary differentiation of endothelial cells from hemangioblasts or angioblasts has long been considered to be a process that is restricted to early embryogenesis, whereas angiogenesis occurs both during development and postnatal life. However, recent studies indicate that the CD34⁺ cell population in bone marrow and peripheral blood from adult humans contains endothelial progenitors (22–24). We have shown that a subset of CD34⁺ stem and progenitor cells expressing the cell-surface marker CD133 has the capacity to differentiate into endothelial cells (25). In addition, Peichev et al. (26) reported that circulating CD34⁺ cells coexpressing CD133 and the vascular endothelial growth factor receptor-2 (VEGFR-2, also known as flk-1/KDR) comprise a functional population of endothelial progenitors. Animal studies suggest that human endothelial progenitors contribute to neovascularization in vivo (27–30). Furthermore, a report by Gunsilius et al. (31) provides strong evidence for the existence of hemangioblasts in adult humans. By culturing bone marrow stem cells from patients with chronic myelogenous leukemia (CML), the investigators demonstrated that *both* hematopoietic and endothelial cells express the pathogenic BCR/ABL fusion gene, the mutation that is restricted to the subset of CML stem cells.

In a series of elegant experiments, Choi's group showed that the embryonic hemangioblast is defined by the expression of VEGF-2 and SCL (16,32,33). Such a definition does not yet exist for the adult hemangioblast. A first study that addressed this question suggests that in the murine system, the adult hemangioblast may display a hematopoietic phenotype (34). Pelosi et al. claimed that in humans, the postnatal hemangioblast is characterized by the expression of CD34 and VEGFR-2 (35). However, no additional information on the expression patterns (e.g., CD133, CD45) of these cells was provided.

In this study, we evaluated the hemangioblastic potential of CD133⁺ cells from mobilized peripheral blood. We show that individual CD133⁺ cells have the capacity to generate both hematopoietic and endothelial cells and that these bipotent precursor cells exhibit a hematopoietic phenotype. This finding has been supported by two recent studies showing that a subset of hematopoietic stem cells can serve as hemangioblasts (see Note Added in Proof, p. 240).

sulin-like growth factor-1 (IGF-1; 10 ng/ml), and leukemia-inhibiting factor (LIF; 1 ng/ml), respectively. All cytokines were from TEBU (Frankfurt, Germany). The combination of VEGF, SCGF, and FLT-3 ligand was determined to increase cell proliferation significantly and was then used for the expansion of the CD133⁺ cells.

Following 4 days in culture under these conditions, 30% of the cells were retrovirally transduced for 2 consecutive days, as described below. At day 6 of culture, transduced cells as well as non-transduced cells were harvested by gentle pipetting. Non-transduced cells were replated at 2×10^6 cells/ml in opaque microtiter well-plates under the same culture conditions. To study the developmental potential of individual CD133⁺ cells, single transduced cells were transferred to cultures with corresponding non-transduced cells from the same sample by limiting dilution, and expanded at the single-cell level for at least six cell doublings. The cultures were incubated at 37°C in 5% CO₂. Additional feeding was performed, depending on cell proliferation. Then, supernatant was removed by gentle pipetting and replaced with fresh medium.

Following 18–28 days of expansion, cells of each well were harvested separately. For endothelial differentiation, one-half of the cells from an individual well were cultured for 14 days in IMDM with 10% FBS, 10% horse serum, 10^{-6} M hydrocortisone, VEGF (50 ng/ml), and SCGF (100 ng/ml). The remaining half was cultured for 7 days in IMDM with 10% FBS, 10% horse serum, 10^{-6} M hydrocortisone, and G-CSF (1000 U/ml) for hematopoietic differentiation. Cells were then harvested for analysis. In parallel experiments, pure cultures with non-transduced cells were performed and analyzed by immunocytochemistry, flow cytometry, and colony assays and in an animal model.

Producer cell lines

The replication-deficient retroviral vector SF11@EGFPprev, a FMEV-based construct encoding the enhanced green fluorescence protein (EGFP) (36), was produced from the PG13 packaging cell line. PG13 cells were kept in Dulbecco's modified Eagle medium (DMEM with glutamax) supplemented with 10% heat-inactivated FBS and 1 mM sodium pyruvate (all from Gibco BRL, Paisley, Scotland). We used X-Vivo 10 (Bio Whittaker, Walkersville, MD) to obtain serum-free vector-containing medium as described (37).

Transduction of CD133⁺ cells

To preload fibronectin-coated, 24-well tissue plates with the vector-containing supernatant, four to six centrifugation steps ($1000 \times g$) in a varifuge 3.0 RS (Heraeus Instruments, Hanau, Germany) at 4°C for 30 min were carried out. Supernatant was discarded after each

centrifugation step. For transduction, we incubated CD133⁺ cells at a density of 2×10^6 cells/ml per well in their expansion medium (as described above) overnight in the preloaded wells without further centrifugation (38). For in vitro studies, two transduction cycles were performed. For in vivo studies, CD133⁺ cells were transduced for 3–5 consecutive days.

Clonogenic assays for multilineage hematopoietic and endothelial progenitor cells

Purified CD133⁺ cells as well as nontransduced cells expanded for 48 days were plated at 5×10^3 cells/ml in methylcellulose (complete media from Cell Systems, St. Katharinen, Germany) as previously described (25). In brief, cultures were stimulated either with a combination of hematopoietic growth factors including: SCF (50 ng/ml), interleukin-3 (IL-3; 20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), GM-CSF (20 ng/ml), plus erythropoietin (3 U/ml) (all from Cell Systems) or with the combination of SCGF (100 ng/ml) and VEGF (50 ng/ml) (both from TEBU). All cultures were performed in duplicate, incubated at 37°C in 5% CO₂ and 95% humidity, and scored after 14 days of culture using an inverted microscope. The absolute number of all types of hematopoietic colonies and of CFU-EC at day 0 and day 14 of culture was calculated by multiplying the number of colonies/ 1×10^3 input cells by the total number of cells present in the respective expansion culture.

Culture and preparation of human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (via Cell Systems, St. Katharinen, Germany) and maintained in EGM-2 (Clonetics). HUVEC were harvested by trypsinization, washed in PBS, and spun at 500 rpm for 5 min onto glass slides by cytocentrifugation.

Immunofluorescence staining of expanded and differentiated cells

Freshly isolated CD133⁺ cells as well as expanded non-transduced cells were spun onto glass slides as described above. Slides were air-dried for at least 10 min and immunostained for the expression of the VEGF receptor 2 (VEGFR2/KDR; Sigma), CD31, von Willebrand factor (vWF), VE-Cadherin (all from Pharmingen), and *Ulex europaeus* agglutinin-1 (Sigma). EGFP⁺ cells cultured for 14 days in expansion medium and for 14 days in differentiation medium were washed twice and spun onto glass slides as described above. After blocking with 10% FBS/RPMI, cytopspins were incubated with the primary antibody for 30 min. The following primary anti-

bodies were used: anti-VE-Cadherin and anti-CD13 (both from Pharmingen, Hamburg, Germany), and *Ulex europaeus* agglutinin-1. This step was followed by incubation with a Texas Red-conjugated goat-anti-mouse secondary antibody (DAKO, Hamburg) for 30 min. Cells were fixed in methanol for 15 min at -20°C and counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Boehringer Mannheim, Germany).

A positive control for the staining with anti-KDR mAb, anti-VE-Cadherin mAb, and *Ulex europaeus* agglutinin-1 was performed with HUVEC. As negative controls, peripheral blood smears as well as the specimen itself were used; these were incubated with a mouse anti-human immunoglobulin (IgG) instead of the primary antibody. Stained cells were examined for the presence of positive cells using a Zeiss Axioplan (Jena, Germany) fluorescence microscope.

Flow cytometry

Isolated CD133^{+} as well as expanded nontransduced cells were washed twice and prepared for two-color flow cytometry. The following PE-conjugated monoclonal antibodies were used: anti-CD133 (Miltenyi Biotec), anti-CD14, anti-CD31, (all from Pharmingen), and anti-c-kit (An der Grub, Kaumberg, Austria). The fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies included: anti-CD34, anti-CD105, anti-CD7, and anti-thy-1 (all from Pharmingen). Isotype-matched mouse Ig served as controls. Cells were incubated with the mAb for 30 min at 4°C . Two-color flow cytometric analysis was performed using a FACS CALIBUR flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson). Each analysis included at least 5000 events. The percentage of CD133^{+} cells present was assessed after correction for the percentage of cells reactive with an isotype control. Using isotype-controls for PE and FITC, gates for phenotypic analysis of CD133^{+} cells were set so that the lower left panel contained at least 98% of the total cells analyzed.

Animal studies

After 14 days of culture in expansion medium and an additional 14 days in differentiation medium, CD133^{+} -derived cells were harvested by trypsinization and washed in PBS before injection into mice. Additionally, cultured tumor cells of the lung cancer cell line A549 (a gift from Prof. K. Havemann, Institute of Molecular Biology and Cancer Research, Marburg, Germany) were prepared, as previously described (25). In parallel experiments, SCID mice were injected subcutaneously with either 1×10^6 tumor cells, with 1×10^6 CD133 -derived putative endothelial cells, or with a mixture of 1×10^6 tumor cells and 1×10^6 CD133 -derived cells. All recipients survived the procedure. At week 5 post-transplan-

tation, all mice were killed for histochemical analysis of the grown tumors.

Immunohistochemical analysis

Tissue blocks of tumors grown in severe combined immunodeficient (SCID) mice following subcutaneous injection of either 1×10^6 A549 lung cancer cells or a mixture of 1×10^6 A549 cells plus 1×10^6 CD133 -derived cells were prepared, as previously described (25). In brief, we used the mAb 4D1/C2 against CEACAM1 together with an amplification combination of the peroxidase anti-peroxidase and the avidin-biotin-peroxidase complex technique. The peroxidase was visualized by use of the Nickel-glucose technique. The specimens were counterstained with Calcium Red. Controls were performed as described (25).

RESULTS

Phenotypic analysis of purified CD133^{+} cells

Flow cytometry was used to characterize the selected CD133^{+} cells (Table 1). The mean purity was $98.3\% \pm 0.3$ (range 95.7–99.9%). Two-color fluorescence analysis revealed a high percentage (mean 97.5%, range 90.6–99.9%) of cells coexpressing the CD34 antigen. As reported earlier (25), small subsets of $\text{CD133}^{+}\text{CD34}^{-}$ cells (0.3–5.1%) were present, whereas $\text{CD133}^{-}\text{CD34}^{+}$ cells were not detected. Purified CD133^{+} cells were also stained for CD90 (thy-1), CD117 (c-kit), CD14, CD31 (PECAM-1), and CD105 (endoglin). Virtually all CD133^{+} cells were CD31^{+} (>99%). CD90^{+} cells ranged from 4.0% to 42.3% and CD117^{+} cells from 1.9% to 27.2%. Very low percentages of CD133^{+} cells expressed the monocytic antigen CD14 (1.5%). To characterize the enriched CD133^{+} populations further, cells were analyzed by immunofluorescence for the presence of KDR and for their ability to bind to *Ulex europaeus* agglutinin-1. The analysis also included the endothelial markers CD31, vWF, and VE-Cadherin. The percentages of positive cells and their staining patterns are shown in Table 2 and Fig. 1. Less than 1% of the CD133^{+} cells expressed the receptor tyrosine kinase KDR. None of the populations assayed stained positively for either *Ulex europaeus* agglutinin-1 or vWF and VE-Cadherin, respectively. In accordance with the findings from flow cytometric analysis, CD31 was expressed on >99% of the purified cells.

Characterization of CD133 -derived cells in expansion culture

Enriched CD133^{+} cells were transferred to fibronectin-coated wells and cultured in the presence of FLT-

FIG. 1. Immunofluorescence analysis of freshly isolated CD133⁺ cells, CD133-derived cells expanded for 14 days, and expanded cells cultured for 14 days in the presence of SCGF and VEGF. (A, D, G, J, and M) Freshly enriched CD133⁺ cells labeled with anti-VE-cadherin monoclonal antibody (MoAb), anti-KDR MoAb, anti-CD31 MoAb, anti-vWF MoAb, and *Ulex europaeus* agglutinin-1, respectively. (B, E, H, K, and N) Staining patterns of CD133-derived cells expanded for 14 days using the same MoAbs. (C, F, I, L, O) Expanded CD133-derived cells cultured for 14 days with SCGF and VEGF stained in the same manner.

TABLE 1. PHENOTYPE OF FRESHLY ISOLATED CD133⁺ CELLS (DAY 0) AND CD133-DERIVED CELLS DURING EXPANSION CULTURE WITH SCGF, VEGF, AND FLT-3 LIGAND

MoAb	Days of culture										
	0	3	6	9	12	14	24	29	35	41	48
CD133	98.3 ± 0.5	81.1 ± 4.1	58.6 ± 20.9	37.6 ± 26.9	8.1 ± 1.5	25.2 ± 7.7	4.48	33.5	61.1	30.3	56.5
CD34	97.8 ± 0.6	91.1 ± 2.5	37.2 ± 5.2	11.5 ± 5.2	4.4 ± 1.9	5.5 ± 1.1	1.0	3.1	6.0	5.7	3.5
CD90	15.2 ± 3.8	25.3 ± 24.4	2.29 ± 1.0	32.7 ± 22.2	24.4 ± 16.1	24.5 ± 9.7	19.3	32.8	26.5	31.3	60.1
CD117	8.2 ± 3.0	24.8 ± 8.1	43.5 ± 13.9	15.0 ± 7.7	2.2 ± 0.7	7.7 ± 2.5	3.4	5.2	15.0	7.1	14.3
CD14	1.5 ± 0.6	2.1 ± 1.2	31.6 ± 9.9	48.9 ± 4.6	40.2 ± 4.4	48.3 ± 6.1	46.3	39.1	28.2	30.6	35.0
CD33	87.1 ± 6.7	83.9 ± 9.5	97.3 ± 1.9	98.4 ± 0.7	97.6 ± 1.1	98.3 ± 0.7	99.4	99.5	94.5	92.6	94.2
CD31	99.4 ± 0.2	99.1 ± 0.8	99.1 ± 0.7	98.0 ± 0.7	97.1 ± 1.9	98.8 ± 0.5	99.6	99.4	99.5	99.7	94.6
CD105	4.2 ± 1.8	8.7 ± 6.9	39.9 ± 22.5	21.5 ± 15.9	36.2 ± 31.1	44.0 ± 16.9	62.0	82.9	61.4	27.3	88.8

Data show analysis by flow cytometry from 9 (day 0), 3 (day 0 to day 14), and 1 (day 0 to day 48) experiments. Values are mean ± SEM.

3 ligand, SCGF, and VEGF. Using these conditions, no significant changes in the morphology of the expanding cells could be observed throughout the culture period. CD133⁺ cells produced a homogeneous loosely adherent population of round cells. Visible proliferation occurred on day 3 of culture. The cell-doubling time varied between 72 and 144 h for the first doubling and was 48–72 h for the following six to eight cell doublings. Thereafter, proliferation seemed to be attenuated. As shown in Fig. 2, proliferation curves showed an up to 72.6-fold increase (mean 37.8-fold) in the total cell number after 14 days, and up to 1087-fold (mean 477-fold) after 31 days of expansion culture. Flow cytometric studies revealed unexpected phenotypic changes of the cells during expansion culture (Table 1). Following a marked decrease in the percentages of cells expressing the CD133 antigen between day 0 and day 12 of culture, this subset again rose within the next two days. On day 48, more than 50% of the cells still expressed CD133. In contrast, the proportion of CD34⁺ cells continuously declined without any significant increase at later timepoints. Although the percentages of CD133⁺ cells and CD34⁺ cells dropped significantly between day 0 and day 6, the sub-

set of cells expressing the SCF receptor c-kit showed a five-fold increase within this period of time. In addition, the CD90⁺ cell fraction expanded two-fold between day 0 and day 9, and remained on this level until day 41. Analysis on day 48 revealed a further increase of this fraction, so that 60% of the cells expressed CD90.

A similar development was observed for the CD105⁺ subset that represented 4.25% of the cells on day 0 and increased up to 88.8% on day 48. The fraction of cells expressing CD14 rose approximately 40-fold until day 9 (mean 48.9%) and subsequently remained on this level. As mentioned before, the vast majority of freshly enriched CD133⁺ cells expressed CD31 prior to culture. This phenotypic characteristic was not altered during the expansion period.

Expanding cells were also stained by immunofluorescence on days 3, 6, 9, 12, and 14 of culture, respectively, for the presence of KDR (Table 2). Until day 9, only few (<1%) KDR⁺ cells were detectable (data not shown). This was consistently demonstrated in three independent experiments. On day 12, KDR⁺ cells ranged from less than 1% to 6.4%, followed by a further increase of KDR⁺ cells on day 14 (4.7–24.0%). None of the cells expanded

TABLE 2. IMMUNOFLUORESCENCE STAINING OF CD133⁺ CELLS AT DIFFERENT DEVELOPMENTAL STAGES

Cells	Antibodies				
	CD31	vWF	CD144	Ulex	KDR
Day 0	>99	0	0	0	<1
Day 6, expanded	N.D.	N.D.	N.D.	N.D.	<1
Day 14, expanded	>99	5.3 ± 1.1	0	0	11.3 ± 6.4
Day 3, differentiated	N.D.	N.D.	N.D.	N.D.	50.5 ± 13.5
Day 9, differentiated	N.D.	N.D.	N.D.	N.D.	82.5 ± 1.5
Day 14, differentiated	>99	>99	>99	>99	87.0 ± 3.4

Abbreviation: N.D., Not done.

for 14 days stained positively for the endothelial markers VE-Cadherin or *Ulex europaeus* agglutinin-1 (Fig. 1 and Table 2).

Taken together, these findings suggest that the expanded population maintained stem cell characteristics with positivity for CD133, CD90, CD117, and KDR, but did not contain mature endothelial cells. In addition to phenotypic analysis, expanding cells were studied for their clonogenic potential. The total number of colonies obtained from cells that were expanded for 14 days (d14 cells) is shown in Fig. 3. In comparison to freshly isolated CD133⁺ cells (d0 cells), d14 cells produced significantly higher numbers of all types of hematopoietic colonies and of CFU-EC due to massive increase in total cell numbers. The increments in the total number of colonies were 3-, 76-, 19-, 43-, and 125-fold for BFU-E, CFU-E, CFU-GEMM, CFU-GM, and CFU-EC, respectively. The relative number of colonies (the number of colonies per 5×10^3 input cells) generated from d14 cells was lower than the number obtained from d0 cells for CFU-GEMM and CFU-GM, which decreased on average 17-fold and 4.5-fold, respectively. The relative number of BFU-E and CFU-E derived from d14 cells showed a high variation. In four of eight experiments, d14 cells produced numbers of BFU-E and CFU-E that were com-

parable to those derived from d0 cells, whereas in the other half of experiments d14 cells did not generate any BFU-E or CFU-E. In contrast, the relative number of CFU-EC derived from d14 cells showed on average a two-fold increase as compared to the number of CFU-EC obtained from d0 cells. This could be demonstrated in all experiments performed. Interestingly, analysis after 40 days of culture demonstrated that the expanded cells still had the capacity to form colonies of the granulocytic/monocytic lineage (data not shown). The absolute number of GFU-GM generated at this timepoint was comparable to the number of CFU-GM produced from day 0 cells (data not shown).

Transduction of expanded CD133-derived cells

During expansion culture, one-third of the CD133-derived cells were retrovirally transduced with the enhanced (E) GFP marker gene. Mean transduction efficiency was 30% (range 9.7–37.9%; Table 3) after two cycles. For in vivo studies, three additional transduction cycles were performed which increased the mean transduction rate to 59.6% (range 34.9–73.4%). In comparison to non-transduced cells, transduction did not alter morphology and growth pattern of expanding cells.

FIG. 2. Proliferation curves of CD133-derived cells in expansion cultures with SCGF, VEGF, and FLT-3 ligand. Expanded cultures were obtained with the supernatant and the number of cells per milliliter was counted. Proliferation rates of six independent experiments followed up to 14 days, and growth curves of three of these six experiments measured until day 31 of expansion culture are shown. Evaluation of the cell count was performed at various timepoints, depending on the individual growth pattern in each experiment. $n = 6$, results from six independent experiments; $n = 3$, results from three independent experiments.

FIG. 3. Clonogenic potential of freshly isolated CD133⁺ cells (day 0 cells) and CD133-derived proliferating cells expanded for 14 days (day 14 cells) in liquid culture with SCGF, VEGF, and FLT-3 ligand. Each experiment was performed in duplicate. The absolute number of all types of hematopoietic colonies and of CFU-EC generated from freshly isolated CD133⁺ cells and from CD133-derived cells expanded for 14 days was calculated by multiplying the number of colonies/ 1×10^3 input cells by the total number of cells present in the respective expansion culture. Day 0 cells, freshly isolated CD133⁺ progenitor cells; day 14 cells, CD133-derived cells cultured for 14 days in liquid culture; BFU-E, burst-forming unit erythrocyte; CFU-E, colony-forming unit erythrocyte; CFU-GEMM, colony-forming unit granulocyte-erythrocyte-macrophage-megakaryocyte; CFU-GM, colony-forming unit granulocyte-macrophage; CFU-EC, colony-forming unit endothelial cell (putative endothelial colonies).

Cytokine-dependent differentiation of CD133⁺ cells expanded at the single-cell level

To study differentiation of CD133⁺ cells at the single-cell level, individual EGFP⁺ cells were plated by means of limiting dilution into wells containing 2×10^6 non-transduced cells/ml to achieve a sufficient cell density for the growth of the marked cells. Using fluorescence microscopy, wells with single EGFP⁺ cells were identified. After 18–28 days (median 26 days) of expansion culture, on average 8% (range 4–25%) of these wells showed at least six cell doublings of the marked cells. Expanded cells from each well were then split and replated. One half was transferred to liquid cultures supplemented with SCGF and VEGF to induce endothelial differentiation. The remaining half of the cells was placed in medium

containing the hematopoietic growth factor G-CSF. In response to SCGF and VEGF, CD133-derived cells became adherent and began to flatten within 1–2 days of culture. Increasing numbers of cells developed an endothelial morphology during a 14-day culture period (Fig. 4A–D). As revealed by immunocytochemistry, these cells expressed VE-Cadherin and stained positive for *Ulex europaeus* agglutinin-1, indicating their endothelial nature (Fig. 4B,D). However, no adherent cells were observed in cultures with G-CSF. After 7 days, these cells displayed characteristics of mature granulocytes with a typical lobulation of the nucleus on DAPI staining and expression of CD13 (Fig. 4E,F). Dual differentiation of single-seeded cells was observed in one quarter of EGFP⁺ clones, suggesting that 2% of the EGFP⁺ cells provided hemangioblast activity.

FIG. 4. Immunofluorescence analysis of CD133-derived cells generated in cultures with single-seeded EGFP⁺ cells. (A,C) Morphology of EGFP⁺ cells cocultured with non-transduced cells for 14 days in expansion medium and for additional 14 days with SCGF and VEGF. (B) Identical cells as shown in A stained with *Ulex europaeus* agglutinin-1. (D) Identical cells as shown in C labeled with VE-Cadherin. (E) Morphology of EGFP⁺ cells expanded in coculture with non-transduced cells for 14 days and differentiated for an additional 14 days in the presence of G-CSF. (F) Identical cells as shown in E labeled with anti-CD13 MoAb. Original magnification, 20 $\times$ (A,B), and 100 $\times$ (C–F), respectively.

FIG. 5. Immunohistochemical staining of subcutaneously induced tumors with the human-specific MoAb 4D1/C2. (A) Negative control (tumor tissue of mice injected with A549 lung cancer cells alone). (B) Negative control (tumor tissue of animals that were inoculated with A549 cells plus CD133-derived putative endothelial cells). (C) Negative staining with 4D1/C2 of blood vessels in tumors initiated with A549 cells alone. (D) Positive staining of blood vessels in tumors grown after subcutaneous injection of both A549 cells and CD133-derived putative endothelial cells. 4D1/C2⁺ tumor cells are indicated by arrows. Positively stained blood vessels are indicated by arrowheads. Original magnification, 100 $\times$.

FIG. 4.

FIG. 5.

TABLE 3. PERCENTAGES OF EGFP⁺ CD133-DERIVED CELLS FOLLOWING RETROVIRAL TRANSDUCTION

Patient	Number of transduction cycles	
	2	5
1	10.1	N.D.
2	22.7	N.D.
3	N.D.	34.9
4	22.1	67.8
5	16.4	62.3
6	18.6	N.D.
7	30.8	N.D.
8	37.9	73.4
9	9.7	N.D.
10	21.8	N.D.
Mean $\pm$ SEM	21.1 $\pm$ 6.0	59.6 $\pm$ 10.4

The percentage of EGFP⁺ cells was determined by flow cytometry.

Abbreviation: N.D., Not done.

In vitro and in vivo characterization of CD133-derived endothelial cells

To further investigate phenotypic changes during differentiation into the endothelial lineage, non-transduced CD133⁺ cells were expanded in bulk cultures for two weeks and then cultured with SCGF and VEGF. Upon withdrawal of FLT-3 ligand, proliferation declined and large adherent cells developed. As early as on day 3 of differentiation culture with SCGF and VEGF, a significant rise of KDR⁺ cells occurred, as detected by immunofluorescence (Table 2). Within the next 10 days, a continuous increase of the KDR⁺ proportion was noted. On day 14, 87% (range 78–91%) of the cells stained positive for this marker. Additional staining for VE-Cadherin, *Ulex europaeus* agglutinin-1, and vWF revealed that virtually all cells expressed these endothelial markers after 14 days of culture, indicating that the vast majority of the cells represented mature endothelial cells.

Xenogenic transplantations were performed to confirm that CD133-derived endothelial cells are functional. SCID mice were injected s.c. with either CD133-derived non-transduced putative endothelial cells, with tumor cells from the lung cancer cell line A549, or with a mixture of CD133-derived cells and tumor cells. Animals that were injected with CD133-derived cells alone did not develop any tumors. Maximal tumor growth was observed in mice that had received both CD133-derived cells and A549 cells. Immunohistochemical analysis of these tumors, using a human specific anti-CEACAM-1 MoAb, revealed positive staining of blood vessels (Fig. 5D), whereas no staining of blood vessel was found in tumors initiated with A549 cells alone (Fig. 5C).

DISCUSSION

In the present study, we investigated whether the human CD133⁺ population from G-CSF-mobilized blood contains precursors that provide hemangioblast activity. Previous studies have shown that human CD133-expressing cells represent a subset of stem and progenitor cells with hematopoietic capacity (39–41). In addition, we and others have demonstrated that CD133⁺ cells can differentiate into the endothelial lineage (25,26). The responsible endothelial precursors within the CD133⁺ population have been identified to coexpress the surface markers CD34 and KDR (26). On the other hand, KDR has been shown to be expressed on human hematopoietic stem cells with multilineage capacity (42). Recently, a study by Pelosi et al. suggested that expression of KDR defines the postnatal hemangioblast (35), as it is the fact in murine embryonic development (15–17). The investigators reported that single KDR⁺ cells could be isolated from mobilized blood by fluorescence-activated cell sorting (FACS) and then cultured in the absence of supplementary cells. However, in the present study less than 0.5% of the freshly isolated CD133⁺ cells (purity >95%) were found to express this marker. These results are essentially consistent with a report by Ziegler et al. (42) and Peichev et al. (26). Because the frequency of CD133⁺/KDR⁺ precursors was less than one in 10,000 of the unselected cell population, we focussed on the total CD133⁺ cells. In our previous experiments, CD133⁺ cells were cultured with SCGF and VEGF to induce endothelial differentiation. Proliferation and survival of the cultured cells under these conditions required high initial cell densities, so that cultures with single CD133⁺ cells could not be performed. To circumvent this problem, we transduced the cells with the EGFP gene and cocultured single marked with non-transduced cells. Using this approach, we demonstrate that cells originating from single CD133⁺ precursors had the capacity to generate both hematopoietic and endothelial cells. Single cells were expanded on average for 24 days before they were transferred to the differentiation cultures. At that time, a significant proportion of the cells was found to express KDR, as determined by analysis of corresponding non-transduced cells. After 2 weeks of culture, portions of these cells could be differentiated into granulocytes and endothelial cells, depending on the culture conditions. These findings support the concept of a bipotent precursor within the CD133⁺ population.

In addition to the KDR⁺ cell fraction, another subset of precursors with hematopoietic and endothelial potential may exist within the CD133⁺ population. Freshly isolated CD133⁺ cells coexpressed several surface markers that are common to both hematopoietic and endothelial cells, such as CD31, and CD105 (6,43,44). More recently, it has been reported that during differentiation of murine

embryonic stem cells CD105 is coexpressed on a subset of mesodermal precursors expressing the VEGFR-2 (45). The investigators suggested that the expression of CD105 characterizes the hematopoietic commitment within the Flk-1⁺ populations. Nevertheless, the authors speculated that the subset Flk-1⁺/CD105⁺ precursors also includes hemangioblasts. In the human system, Pierelli et al. (44) showed that subsets of circulating CD34⁺ cells expressing CD105 are enriched in primitive hematopoietic precursors. In bone marrow and cord blood, this subset contained all CD34⁺/CD38⁻ precursors that are suggested to represent immature stem cells with hematopoietic potential (46,47). Whether human CD34⁺/CD38⁻/CD105⁺ cells can give rise to cells of the endothelial lineage is unknown.

In addition, expression of CD90 was previously supposed to be restricted to primitive hematopoietic cells (48,49). Recently, it has been reported that CD90 is also expressed on adult vascular endothelium in mice (50,51). We have found more than 20% CD90⁺ cells within the expanded population throughout the culture period. One possible explanation for this finding is that the culture conditions used were optimal to support the growth of primitive hematopoietic precursors. Alternatively, expression of CD90 could be a marker for hemangioblasts.

Furthermore, we observed up to 54% of CD14⁺ cells after 14 days of culture. CD14 that has been widely accepted as a marker specific for monocytes (52). Two recent studies have revealed expression of CD14 on human endothelial cells (53,54). Moreover, it has been suggested that mature monocytes can transdifferentiate into endothelial cells (55–58). Hence, we cannot exclude that the endothelial cells generated from single EGFP⁺ precursors developed via transdifferentiation of CD14⁺ cells.

To accomplish efficient transduction of the CD133⁺ cells, we developed a culture system for the ex vivo expansion of these precursors. Past studies have shown that in the ex vivo expansion systems, proliferation usually occurs with differentiation and loss of stem and progenitor cell activity (59–61). Recently, it has been demonstrated that self-renewal of hematopoietic stem cells can be promoted using a combination of early cytokines, such as FLT-3 ligand, SCF, and thrombopoietin (62). We used the combination of FLT-3 ligand, SCGF, and VEGF, hypothesizing that this cocktail may have the ability to maintain the hematopoietic as well as the endothelial potential of the CD133⁺ precursors. While stimulation with SCGF and VEGF mainly results in endothelial differentiation of the CD133⁺ population (25), the addition FLT-3 ligand was found to induce high proliferation for an extended culture period. Analysis of the expanded cells revealed several unexpected phenotypic changes. Following a progressive decline from day 0 to day 12 of culture, the proportion of CD133⁺ increased again thereafter. Interestingly, the fraction of cells coexpressing CD34 cells decreased in parallel without any significant increase at

later timepoints. Consequently, the culture conditions used supported the growth of CD133⁺/CD34⁻ cells.

Whether these expanded CD133⁺/CD34⁻ cells have the primitive stem cell properties as the small subset of CD133⁺/CD34⁻ cells that can be found in freshly isolated CD133⁺ populations (63) remains to be determined. The observation that the proportion of CD90⁺ cells also increased throughout the culture period leads us to speculate that the expanded CD133⁺/CD34⁻ population has stem cell activity. In accordance with this interpretation, we could demonstrate that the proportion of precursors with clonogenic potential significantly expanded after 14 days of culture. The increase in the number of colonies was most pronounced for CFU-EC. Together with findings from immunocytochemical analysis, these data suggest that the endothelial colonies derive from KDR⁺ cells that might be selectively expanded in the VEGF-containing culture medium.

Interestingly, after 40 days of culture expanded cells still had the capacity to form hematopoietic colonies, whereas the ability to generate endothelial colonies was lost. On the other hand, our experiments at the single-cell level demonstrated CD133-derived cells could be expanded for up to 28 days without losing the potential to differentiate into both hematopoietic and endothelial cells. Hence, our culture system may be of great clinical relevance in the development of protocols for therapy with ex vivo expanded hematopoietic and endothelial precursors. Furthermore, the presented culture conditions are suitable for an efficient and stable transduction of CD133⁺ stem and progenitor cells, including those with hemangioblastic potential. Transduction did not alter the proliferative or the differentiation potential of the expanded cells. In vivo analysis revealed that the EGFP-transgene was expressed for at least 2 months. The findings from our study may also be useful in the development of novel strategies for gene therapy of genetic, malignant, and vascular diseases.

In conclusion, we identified a subset of CD133⁺ cells that have the capacity to generate both hematopoietic and endothelial cells and, therefore, fit the description of the hemangioblast. These precursors cells certainly show therapeutic promise, but their physiological relevance is not yet clear. Are these cells involved in maintaining hematopoiesis and vasculogenesis in adults? Work is in progress to address this important issue and to determine if these cells satisfy the stringent criteria defined by Choi and colleagues, whose studies have pioneered the definition of the elusive hemangioblast (64).

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Note Added in Proof:

After this work was accepted for publication, two independent transplantation studies have claimed identification of the postnatal human hemangioblast (Cogley CR et al., Blood 103:133–135, 2004 and Bailey AS et al., Blood 103:13–19, 2004). Both confirm our observations that this bipotent precursor represents a subset of CD34⁺ bone marrow-derived hematopoietic stem cells with the capacity to contribute to both hematopoietic reconstitution and blood vessel formation.

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