JCI The Journal of Clinical Investigation

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J Clin Invest. 2001;108(7):1071-1077. https://doi.org/10.1172/JCI13297.

Article

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Transplantable hematopoietic stem cells in human fetal liver have a CD34$^+$ side population (SP) phenotype

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Received for publication May 17, 2001, and accepted in revised form August 21, 2001.

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J. Clin. Invest. **108**:1071–1077 (2001). DOI:10.1172/JCI200113297.

Introduction

Identification of molecular markers that characterize transplantable human cells with stem cell potential and that allow their selective purification remains an important prerequisite for many applied as well as basic studies. This has been recently underscored by reports that hematopoietic stem cells (HSCs) can generate other types of tissue progeny to correct damage to organs including the liver, skeletal muscle, and heart (1–3). The first and still most widely used marker for purifying human HSCs is the CD34 sialomucin antigen (4). Highly enriched populations of human cells expressing CD34$^+$ reconstitute multilineage hematopoiesis not only in sublethally irradiated immunodeficient mice (5, 6), but also in human recipients (7–10). Initially, it was assumed that in humans, only cells expressing CD34 would display HSC activity, as most types of primitive hematopoietic cells appeared to be exclusively CD34$^+$ (11, 12), and the frequency of CD34$^+$ cells is now commonly used to anticipate the adequacy of clinical hematopoietic cell transplants. Recently, however, several groups including ours have provided evidence of various types of primitive human hematopoietic progenitors that do not express detectable levels of CD34 (13–17). On the other hand, studies in mice indicate that CD34 expression may be subject to modulation on HSCs, as it is expressed on murine HSCs during development (18, 19) but not in the adult (20), unless homeostasis is perturbed (21).

In 1996, Goodell et al. reported a new method of obtaining enriched populations of HSCs from adult mouse bone marrow in a single step (22). This procedure exploits the ability of HSCs to efflux the fluorescent dye Hoechst 33342, which, like the activity of P-glycoprotein (encoded by the *MDR* gene), is verapamil-sensitive (23). The Hoechst 33342^{lo} cells thus isolated were called side population (SP) cells (22) and, not surprisingly, were found to have the same CD34$^-$ Sca-1$^+$ lineage marker-negative (lin$^-$) phenotype independently established for HSCs in adult mouse bone marrow (20, 24, 25). SP cells have since been identified in adult bone marrow from several species (including humans) (26). To date, a description of the functional activities of human SP cells in normal individuals has been limited to a single in vitro study of cord blood cells that showed a partitioning of the myeloid and NK (lymphoid) progenitor activities in the CD34$^+$ and CD34$^-$ subsets of the SP cells (27). We now demonstrate that SP cells are already present in the second-trimester human fetal liver. There they constitute a phenotypically and functionally heterogeneous population but, importantly, contain all of the cells able to repopulate nonobese diabetic/LtSz-*scid/scid* (NOD/SCID) mice and only a minor subset of progenitors detectable by available in vitro assays. The present studies also provide the first evidence to our knowledge that transplantable HSCs in human fetal liver, like those present in the early mouse embryo (18), are exclusively CD34$^+$, suggesting a possible parallelism in the regulation of this surface marker on human HSCs.

Table 1
Comparison of antigens expressed on the $CD34^+$ and $CD34^-$ subsets of SP cells present in the low-density fraction of human fetal liver cells

Antigen analyzed	Percent of antigen$^+$ cells in each SP fraction	
	$CD34^+$ (23% ± 10% of all SP cells)	$CD34^-$ (78% ± 10% of all SP cells)
CD2	4 ± 3	13 ± 4
CD3	0 ± 0	0 ± 0
CD7	0 ± 0	36 ± 14
CD14	0 ± 0	0 ± 0
CD16	0 ± 0	0 ± 0
CD19	0 ± 0	0 ± 0
CD33	64 ± 27	6 ± 2
CD36	12 ± 3	37 ± 19
CD38	22 ± 4	40 ± 15
CD45	77 ± 16	44 ± 16
CD56	4 ± 3	1 ± 1
CD66b	0 ± 0	0 ± 0
CD71	31 ± 9	43 ± 16
CD117	62 ± 18	9 ± 3
Glycophorin A	5 ± 2	43 ± 16

Values represent the mean ± SEM from three experiments.

Methods

Human cells. Human fetal livers were obtained from 14- to 21-week-old aborted fetuses according to approved institutional procedures. Single-cell suspensions were prepared using dispase (Sigma Chemical Co., St. Louis, Missouri, USA) (28), and the cells were then usually cryopreserved at –135°C. For initial studies of lineage (lin) marker expression on fetal liver SP cells, low-density (<1.077 g/ml) cells were first isolated by centrifugation on Ficoll-Hypaque (Pharmacia Biotech A/B, Uppsala, Sweden). However, for later progenitor assays, this first step was omitted, which enabled higher yields of SP cells to be obtained after the removal of lin^+ cells (data not shown).

Animals. NOD/SCID mice (29) were bred and maintained under defined sterile conditions in our animal facility. At 6–8 weeks of age, they were irradiated with 350 cGy of ^{137}Cs γ-rays and then injected intravenously the next day with human test cells (as indicated) plus 10^6 irradiated (1,500 cGy) human bone marrow carrier cells.

Cell purification. Human fetal liver cells were thawed and depleted of lin^+ cells using a StemSep column (StemCell Technologies, Vancouver, British Columbia, Canada) according to the supplier's directions after staining the cells with the following cocktail of mAb's: CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A. The lin^- cells collected after passage of the cells through the column were kept overnight at 4°C in Iscove's medium supplemented with a serum substitute (BIT 9500; StemCell Technologies) plus 40 μg/ml of human low-density lipoprotein (Sigma Chemical Co.) and were then incubated with 5 μg/ml Hoechst 33342 (Sigma Chemical Co.) at 37°C for 90 minutes at 10^6 cells/ml. A parallel aliquot was stained with the Hoechst dye in the presence of 50 μM verapamil (Sigma Chemical Co.). Cells were then washed, resuspended in ice-cold HBSS containing 2% FBS (HF; StemCell Technologies) plus 5% pooled normal human serum (Sigma Chemical Co.) (HF/5% HS) at 10^7 cells/ml, and then stained for 30 minutes on ice with various mAb's: FITC-conjugated CD34 (8G12; kindly provided by P.M. Lansdorp, Terry Fox Laboratory, Vancouver, British Columbia, Canada; ref. 30), CD3 (Leu-4), CD7 (Leu-9), CD14 (MϕP9), CD16 (NKP15), and CD66b (G10F5), and PE-conjugated CD2 (Leu-5b), CD19 (Leu-12), CD33 (P67.6), CD34 (anti–HPCA-2), CD36 (CB38), CD38 (HB7), CD45 (HI30), CD56 (Leu-19), all from Becton Dickinson Immunocytometry Systems (San Jose, California, USA), and CD71 and glycophorin A (OKT9 and 10F7, respectively, kindly provided by P.M. Lansdorp) either for analysis or sorting. Stained cells were washed once in HF and then again in HF with 2 μg/ml propidium iodide (PI; Sigma Chemical Co.). Cells were analyzed on a FACSort using CellQuest software and were sorted on a dual laser FACStar$^+$ (all, Becton Dickinson Immunocytometry Systems). Hoechst 33342 was excited at 360 nm, and fluorescence emission was detected using 424/BP44 and 660/BP20 optical filters (Omega Optical Inc., Brattleboro, Vermont, USA) and a 640-nm short-pass dichroic mirror (Omega Optical Inc.) to separate emission wavelengths.

Progenitor assays. Cells were plated in methylcellulose medium (MethoCult H4230; StemCell Technologies) with 3 U/ml human erythropoietin (StemCell Technologies), 50 ng/ml of recombinant human Steel factor (SF; prepared in the Terry Fox Laboratory), and 20 ng/ml each of human IL-3 (Novartis, Basel, Switzerland), human IL-6 (Cangene, Mississauga, Ontario, Canada.), human G-CSF (StemCell Technologies), and human GM-CSF (Novartis) to assess their direct granulopoietic, erythropoietic, and multilineage colony-forming cell (CFC) content, as described elsewhere (31). For long-term culture-initiating cell (LTC-IC) assays, test cells were first cocultured for 6 weeks at 37°C with preestablished, irradiated feeder layers of mouse fibroblasts engineered to produce human IL-3, G-CSF, and SF (31). At the end of 6 weeks, a single-cell suspension was prepared from the whole culture and assayed for its CFC content.

Measurements of human cells able to repopulate NOD/SCID mice (competitive repopulating units or CRUs). Bone marrow cells were harvested from the femurs of primary and secondary mice at the times indicated. The bone marrow cells were suspended in HF/5% HS and then first incubated for 10 minutes at 4°C with HF/5% HS supplemented with 3 mg/ml of an antimouse Fc receptor antibody (2.4G2) to block Fc receptors and prevent subsequent nonspecific antibody binding (32). The cells were then stained for 30 minutes at 4°C with antihuman CD34-FITC, CD19-PE, and CD20-PE (Becton Dickinson Immunocytometry Systems) or antihuman CD71-PE, CD45-PE, CD15-FITC, and CD66b-FITC as described previously (33, 34). Various phenotypes within the viable (PI^-) fraction were then determined. A mouse was considered positive for a given population if, and only if, more than five of

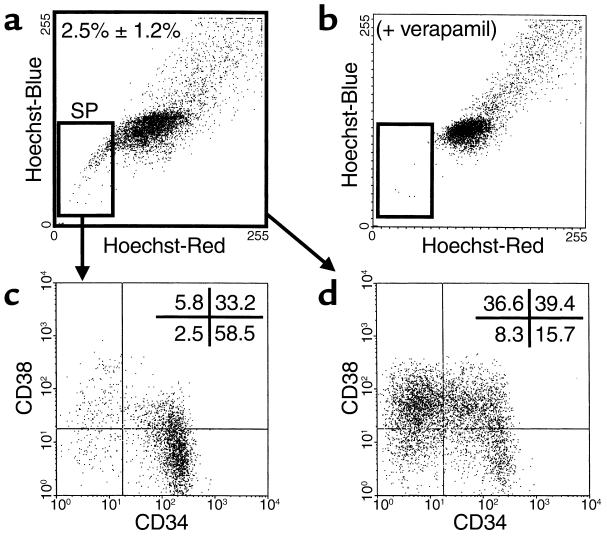

Figure 1
Representative FACS dot plot showing the presence and phenotypes of SP cells in the lin⁻ fraction of human fetal liver cells. Cells were depleted of lin⁺ cells and stained with Hoechst 33342 and antibodies to CD34 and CD38 as described in Methods. (**a**) Small gated cell population identifies the SP cells (2.5% ± 1.2% of the total lin⁻ fetal liver population; $n = 9$) that disappear in the presence of verapamil (**b**). The distribution of cells according to their expression of CD34 and CD38 in the SP (**c**) and total (**d**) fractions of the lin⁻ fetal liver population analyzed here is also shown.

20,000 events analyzed had a fluorescence greater than that exhibited by at least 99.99% of the cells stained with an irrelevant isotype-matched control antibody labeled with the corresponding fluorochrome. A mouse was considered positive for human CRU engraftment if, and only if, it was positive for both human lymphoid and human myeloid cells (33, 35). CRU frequencies were calculated with the L-calc software program (StemCell Technologies) that uses Poisson statistics and the method of maximum likelihood.

Statistical analysis. Significant differences ($P < 0.05$) were established using the Student's *t* test.

Results

SP cells in human fetal liver are phenotypically heterogeneous. Initial Hoechst 33342 staining of low-density human fetal liver cells showed the SP fraction to be small (0.14% ± 0.07%; $n = 3$), but readily detectable. Further analyses revealed these fetal liver SP cells to contain a heterogeneous mixture of phenotypes (Table 1). About 80% were CD34⁻, most of which expressed at least one of the various lin markers evaluated. The most prominent of these appeared to be members of the erythroid and lymphoid lineages based on their expression of glycophorin A (a marker of relatively mature erythroid precursors) or CD7 (a marker typical of some lymphoid precursors). The high numbers of SP cells found to express CD36 and CD71 (markers also expressed at elevated levels on early erythroid cells) and CD38 (present on many early lymphoid cells) are consistent with the data for glycophorin A and CD7. Within the remaining approximately 20% of low-density SP fetal liver cells that were CD34⁺, the most prominent lin⁺ subset expressed the granulopoietic marker CD33. However, 5% of the CD34⁺ SP cells were glycophorin A⁺, indicating the presence of erythroid precursors within this subset also. CD45, a marker expressed on all hematopoietic cells except for terminally differentiating erythroblasts, was found on 77% and 44% of the CD34⁺ and CD34⁻ subsets, respectively, of the low-density SP fetal liver cells (Table 1). The higher numbers of CD45⁻ cells in the CD34⁻ fraction of SP cells are consistent with these being late erythroid cells, as also indicated by removal of many of the CD45⁻ cells in the lin⁺ cell depletion step (data not shown).

Initial studies indicated that centrifugation on Ficoll-Hypaque reduced the yield of SP cells approximately twofold (data not shown). Therefore, subsequent studies to determine which progenitors express an SP phenotype were undertaken with lin⁻ fetal liver cells (cells not expressing CD2, 3, 14, 16, 19, 24, 56, 66b, or glycophorin A) that had not been subjected to a density centrifugation procedure. Removal of these lin⁺ cells increased the proportion of SP cells in the population thus obtained to 2.5% ± 1.2% ($n = 9$; Figure 1, a and b), as expected from the phenotyping analyses (Table 1). The proportion of lin⁻ cells that expressed CD34 was also markedly and selectively increased to 78% ± 6% (vs. 23% ± 13%; Table 1) as was the subset of lin⁻ SP cells that possessed the CD34⁺CD38⁻ phenotype associated with transplantable stem cell activity in cord blood (5, 33) (from 18% ± 11% [Table 1] to 62% ± 6% [$n = 9$; see also Figure 1, c and d]).

Very few progenitors in human fetal liver that are detected by in vitro assays have a lin⁻ CD34⁺CD38⁻ SP phenotype. Table 2 shows the numbers of progenitors detectable by both short-term clonogenic and long-term stromal-based coculture assays detected per hundred total or SP⁺ CD34⁺CD38⁻ lin⁻ fetal liver cells. Interestingly, both the frequency of clonogenic progenitors (CFCs) in the SP fraction of the CD34⁺CD38⁻ lin⁻ fetal liver cells and

Table 2
Frequencies of total and different types of CFCs and of LTC-ICs in the total and SP fraction of CD34⁺CD38⁻ and CD34⁻CD38⁻ lin⁻ human fetal liver cells

Phenotype[A]	CFC per 100 cells[B]				LTC-IC per 100 cells[B,C]
	Total	CFU-GM	BFU-E	CFU-GEMM	
CD34⁺CD38⁻-SP	17 ± 6	16 ± 7	0 ± 0	2 ± 1	470 ± 330
CD34⁺CD38⁻-total	16 ± 7	14 ± 8	0 ± 0	2 ± 2	690 ± 390
CD34⁻CD38⁻-SP	2.4 ± 1.4	0.5 ± 0.6	0.2 ± 0.2	1.8 ± 0.9	0
CD34⁻CD38⁻-total	5.8 ± 2.2	1.0 ± 0.6	0.8 ± 0.5	4.0 ± 1.2	0

[A]All cells were exposed to Hoechst 33342. [B]Values shown are the mean ± SEM from three experiments. [C]Data shown represent the total number of CFCs measured in the cells harvested from 6-week LTC assay cultures initiated with 400 cells of each population tested.

Table 3
NOD/SCID engraftment data for the total and SP subset of $CD34^+CD38^-$ and $CD34^-CD38^-$ lin^- human fetal liver cells

Phenotype[A]	Prior culture[B]	Cells per mouse[C] ($\times 10^3$)	Positive mice/ total[D]	Percent human cells in positive mice			CRU frequency[E] ($\times 10^{-3}$)
				Total[F]	Myeloid[F]	Lymphoid[F]	
$CD34^+CD38^-$-SP	No	22.2	2/2	79	5	45	1/3.4
		4.5	0/2	<0.025			(1/2.2–1/5.4)
		1.0	3/10	2–65	0.07–5	1–38	
		0.6	1/2	0.1	0.03	0.1	
		0.3	2/9	3	0.4	1	
$CD34^+CD38^-$-total	No	200–1,400	5/5	53–96	4–9	23–73	1/39
		100–200	16/18	6–86	0.3–12	3–57	(1/32–1/49)
		20-60	13/16	0.3–60	0.03–9	0.2–36	
		5	8/19	0.5–64	0.04–12	0.3–17	
$CD34^-CD38^-$-SP	No	5–10	0/4	<0.025			<1/25[H]
	Yes	1–1.8	0/2	<0.025			(1/9.4–1/67)
	Yes[G]	3-15	0/3	<0.025			
$CD34^-CD38^-$-total	No	180–1400	0/3	<0.025			<1/1,100[H]
	Yes	75–110	0/2	<0.025			(1/420–1/2,900)
	Yes[G]	25–280	0/3	<0.025			

[A]All cells were exposed to Hoechst 33342. [B]Cells were cultured for 3–4 days in serum-free media with human flt-3 ligand (100 ng/ml; Immunex Corp., Seattle, Washington, USA), SF (100 ng/ml), IL-3 (20 ng/ml), hyper–IL-6 (10 ng/ml; kindly provided by S. Rose-John, University of Kiel, Kiel, Germany), and thrombopoietin (TPO, 50 ng/ml; Genentech Inc., South San Francisco, California, USA), or with HUVEC feeders (obtained from the American Type Culture Collection, Rockville, Maryland, USA) as described elsewhere (13). [C]Cell number expressed in preculture starting equivalents. [D]Positive mice were defined as those whose bone marrow cells contained more than five human lymphoid and more than five human myeloid cells in 20,000 cells analyzed as described in Methods. [E]Values shown in parentheses are the upper and lower limits of the range defined by ± SEM. [F]Total, myeloid, and lymphoid are defined as $CD45/71^+$ cells, $CD45/71^+CD15/66b^+$ cells, and $CD34^-CD19/20^+$ cells, respectively. [G]Cells were initially depleted of $CD19^+$ and glycophorin A^+ cells only. [H]These upper estimates were calculated assuming one mouse given the maximum number of cells tested had been positive and serve to indicate the sensitivity of the assay in this instance.

the types of colonies they produced were indistinguishable ($P > 0.05$) from those generated by the total $CD34^+CD38^-$ lin^- cell population (> 80% granulopoietic, with the remainder being multilineage). The frequency of the more primitive LTC-ICs in the total and SP subset of $CD34^+CD38^-$ lin^- fetal liver cells was also similar (~7% and 10%, respectively, assuming both will have produced, on average, 72 clonogenic progenitors per LTC-IC at the 6-week time point when their CFC output activity was assessed; ref. 36). Thus, isolation of the SP subset within the $CD34^+CD38^-$ lin^- population did not enrich further for LTC-ICs. Nevertheless, the frequency of LTC-ICs obtained here for the $CD34^+CD38^-$ lin^- cells was seven to tenfold higher than the values that we have previously reported for the total (low-density) lin^- fetal liver cell population (36).

All of the transplantable human hematopoietic stem cells present in human fetal liver have a lin^- SP^+ $CD34^+CD38^-$ phenotype. We next compared the frequency of human cells in the SP versus the total lin^- $CD34^+CD38^-$ fraction that were able to regenerate both lymphoid and myeloid progeny in transplanted NOD/SCID mice. Varying numbers of total $CD34^+CD38^-$ lin^- fetal liver cells and the SP subset of these were injected into sublethally irradiated NOD/SCID mice in a total of 13 and four experiments, respectively. Six to 24 weeks later, the mice were assessed for the presence of human lymphoid (B-lineage markers) and human myeloid (granulopoietic) cells in their bone marrow as described in Methods. The results are shown in Table 3. In contrast to the results for the progenitors measured by both short- and long-term in vitro assays, the CRU activity was exclusively associated with the SP subset of the $CD34^+CD38^-$ lin^- population. Most of the positive mice were highly repopulated with both lymphoid and myeloid human cells; however, some mice were not. Accordingly, Poisson statistics could be used to calculate the frequencies of transplantable human stem cells (CRUs) in the original populations transplanted, and these showed an approximately tenfold enrichment in the frequency of CRU in the SP subset of the $CD34^+CD38^-$ population. However, the absolute value obtained here for the frequency of CRU in the $CD34^+CD38^-$ lin^- fetal liver cell population is somewhat lower than what we anticipated based on our previous data for lin^- fetal liver cells (35). This was found in separate studies to be due in part to an approximately threefold loss of CRU from the toxic effect of exposing the cells to Hoechst 33342, and in part to the lack of removal in the present study of cells with a density >1.077 g/ml (which increased CRU yields but also decreased their final frequency in the lin^- $CD34^+CD38^-$ SP cells another twofold; data not shown).

Transplantation of NOD/SCID mice with cells from the non-SP fraction of the $CD34^+CD38^-$ lin^- population failed to detect CRUs in this fraction at a frequency that could not be explained by contamination by SP cells, i.e., in a total of six experiments, in which six mice were injected with 15×10^3 to 285×10^3 non-SP $CD34^+CD38^-$ lin^- fetal liver cells, only a single positive mouse was detected when these were analyzed 12 weeks later, and this was the mouse that received the largest number of non-SP cells.

To assess the in vivo self-renewal activity of the CRUs isolated in the SP fraction of the $CD34^+CD38^-$ lin^- fetal liver cells, bone marrow cells from both femurs and tibias of three highly engrafted primary recipients (containing >30% human cells) from two different experiments were harvested 7–8 weeks after transplant and

injected individually into three secondary NOD/SCID mice. In each of these secondary mice, a large number of human lymphoid and myeloid progeny was detected 6–8 weeks after transplant ($CD45/71^+$: 86%, 90%, 76%; $CD45/71^+CD15/66b^+$: 5%, 6%, 7%; and $CD34^-CD19/20^+$: 42%, 68%, 59%). Results for one of these is shown in Figure 2. These findings are similar to what we have seen previously in secondary recipients of cells from mice originally transplanted with unseparated lin^- human fetal liver cells (35).

Human fetal liver contains some primitive progenitors that do not express CD34, but these are not detectable in long-term culture assays and do not engraft NOD/SCID mice. As illustrated in Figure 1, only a small proportion of the SP lin^- cells were $CD34^-CD38^-$ (6.4% ± 1.4%; $n = 9$), and these comprised only 0.8% ± 0.4% ($n = 9$) of the total $CD34^-CD38^-$ lin^- population. The results of assays for CFCs and LTC-ICs in the total and SP subset of the $CD34^-CD38^-$ lin^- population are given in Table 2, and for CRUs in Table 3. The frequency of CFCs in the total $CD34^-CD38^-$ lin^- population was two- to threefold lower than in the total $CD34^+CD38^-$ lin^- population. The $CD34^-CD38^-$ CFCs were also made up of a significantly different ($P < 0.05$) spectrum of CFC types; i.e. they contained a much higher proportion of multilineage CFCs and many fewer granulopoietic CFCs. Moreover, although isolation of the SP subset of $CD34^-CD38^-$ cells resulted in a two to threefold reduction in CFC frequency, the distribution of progenitor subtypes in the SP fraction of the $CD34^-CD38^-$ population did not change. Neither LTC-ICs nor CRUs could be detected in either the total or the SP subset of $CD34^-CD38^-$ lin^- fetal liver cells in any of three to four experiments, even when these were assayed at the same or much larger numbers than were sufficient to detect such activities in the corresponding subsets of $CD34^+$ cells. Failure to detect CRU activity in any of the $CD34^-CD38^-$ cells also included experiments in which these were first cultured for 3–4 days with human growth factors or on top of human umbilical vein endothelial cell (HUVEC) feeders under conditions reported to stimulate the generation of NOD/SCID repopulating cells from $CD34^-$ cord blood cells (13).

Discussion

We show here that cells able to efflux Hoechst 33342 and thus designated as SP cells are readily detectable in human fetal liver and comprise a heterogeneous mixture of cell types as indicated by their surface marker profile, as previously established for cells in other hematopoietic tissues of human or nonhuman origin (26, 27). That the SP phenotype has been reported to be broadly associated with in vivo HSC activity (22, 26) and with stem cell activity for other tissues (2, 37) prompted us to investigate its association with human HSC properties. For this, we measured the ability of the various fractions of human fetal liver cells isolated to engraft sublethally irradiated NOD/SCID mice with both lymphoid and myeloid progeny when assayed at limiting doses (i.e., doses of cells that give no human cell engraftment of some mice). We also examined their in vivo self-renewal activity using secondary transplants (6, 34, 35). Our results show that all of the transplantable HSCs present in the $CD34^+CD38^-$ lin^- population of the second-trimester human fetal liver are contained within the SP fraction. This provides the first evidence of a human stem cell population that shares this phenotype and predicts the future extension of these observations to other human tissues both during development and in the adult.

We focused here on the $CD34^+CD38^-$ lin^- subset because previous studies had indicated most of the human HSCs able to engraft NOD/SCID mice have this phenotype (5, 33). We found that only about 10% of the $CD34^+CD38^-$ lin^- cells in human fetal liver have an SP phenotype, and isolation of these SP cells resulted in a corresponding enrichment in the transplantable HSC content of this subset. Accordingly, it would be expected that this additional purification step would allow a more highly enriched population of transplantable HSCs to be isolated than has been previously attainable, which would constitute a major advance for future biologic and molecular analyses. Unfortunately this expectation was not realized here because of the inherent toxicity of exposing the cells to Hoechst 33342 and the elimination of an initial low-density centrifugation step in order to improve the final yield of SP cells. Recently, it was reported that the SP phenotype is due to the selective expression on stem cells of ABC-G2/BCRP (38, 39), which, like the MDR/P-glycoprotein (23), is a verapamil-sensitive member of the ABC transporter family (40–42) and is likewise expressed on the cell surface (43, 44). Thus, in the future it may be possible for SP cells to be isolated using nontoxic, specific antibody-based procedures.

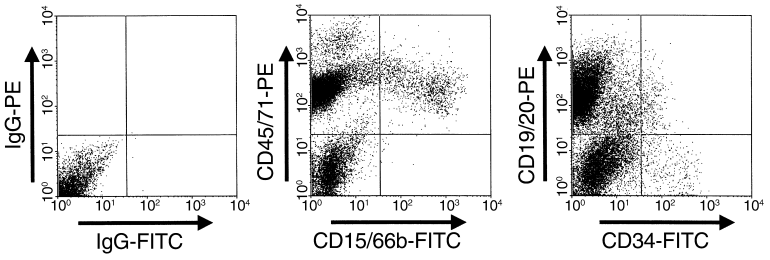

Figure 2
Representative FACS dot plot showing human multilineage engraftment in a secondary NOD/SCID recipient of initially transplanted SP fetal liver cells. The bone marrow cells were removed from both femurs and both tibias of a first NOD/SCID mouse transplanted 7 weeks previously with 22,000 SP $CD34^+CD38^-$ lin^- human fetal liver cells, and all of these cells (~25% of the total bone marrow of that mouse [ref. 48], of which 76% were human) were then transplanted into a second NOD/SCID mouse. The analysis shown is for the bone marrow cells removed from the secondary mouse another 6 weeks later.

Our findings with $CD34^+CD38^-$ lin^- SP fetal liver cells provide new evidence that cells detected using in vitro stromal-based cultures, even after prolonged periods in the presence of increased level of human growth factors (31, 45), cannot necessarily be equated with cells able to serially engraft NOD/SCID mice or fetal sheep (35, 46). This was demonstrated in the present studies by the nonselective partitioning of LTC-ICs seen between the SP and non-SP subsets of the $CD34^+CD38^-$ lin^- cells that contrasted with the complete segregation of the transplantable HSC activity in the SP fraction. Such findings suggest that less than 10% of the LTC-ICs identified in human fetal liver with the 6-week assay used here (31) would have transplantable HSC activity. These findings underscore the need for similar comparative studies of SP and non-SP subsets from other sources of human cells with in vitro and in vivo hematopoietic activity.

In marked contrast to the results for $CD34^+CD38^-$ lin^- fetal liver cells, we did not detect any in vivo reconstituting activity in the corresponding $CD34^-$ population of human fetal liver cells. Although this finding appears to contrast with previously reported results for adult and neonatal sources of transplantable human hematopoietic cells (13, 14, 16, 17), this apparent discrepancy may be due to the different ontological status of the cells being compared, as CD34 expression on transplantable murine HSCs has been found to change during development (19). In addition, our findings do not exclude the possibility that $CD34^-$ progenitors restricted to the T cell and/or NK lineages may exist in human fetal liver since differentiation of human cells along these lineages has not been seen in NOD/SCID mice. The observation that all transplantable cells in human fetal liver that have B-lineage and myelopoietic potential are $CD34^+$ is thus consistent with the concept that CD34 expression may be altered in HSCs according to their activation status, as first indicated by the finding that CD34 expression in $CD34^-$ lin^- hematopoietic cells can be upregulated by exposure to certain feeders or growth factors in vitro, or after their stimulation in vivo (13, 15, 17, 21, 26). We have recently demonstrated differences in the level of expression of several other genes in freshly isolated $CD34^+CD38^-$ lin^- cells from human fetal liver compared with cord blood or adult bone marrow (47). Moreover, some of these changes in gene expression are also triggered by growth factor activation of adult human bone marrow $CD34^+CD38^-$ lin^- cells even before their exit from G_0. Taken together, our present findings predict that the SP phenotype will be an important marker of human cells with stem cell potential and may be more consistent than expression of CD34.

Acknowledgments

We thank G. Thornbury and R. Zapf for assistance in cell sorting; members of the Stem Cell Assay Service for performing some of the cell separations; A. Ahamed for help in preparing the manuscript; and P. Lansdorp, S. Rose-John, Cangene, Genentech, Immunex, Novartis, and StemCell for valuable gifts of reagents. This work was supported by an NIH grant (NHLBI PO1 55435) and by the National Cancer Institute of Canada (NCIC) with funds from the Terry Fox Run and the Canadian Cancer Society. N. Uchida was supported by funds from Kirin Brewery Inc. and a Canadian Institutes of Health Research Postdoctoral Fellowship. C. Eaves was a Terry Fox Cancer Research Scientist of the NCIC.

1. Lagasse, E., et al. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nat. Med.* **6**:1229–1234.
2. Gussoni, E., et al. 1999. Dystrophin expression in the *mdx* mouse restored by stem cell transplantation. *Nature.* **401**:390–394.
3. Orlic, D., et al. 2001. Bone marrow cells regenerate infarcted myocardium. *Nature.* **410**:701–705.
4. Krause, D.S., et al. 1994. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. *Blood.* **84**:691–701.
5. Bhatia, M., Wang, J.C.Y., Kapp, U., Bonnet, D., and Dick, J.E. 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc. Natl. Acad. Sci. USA.* **94**:5320–5325.
6. Hogan, C.J., et al. 1997. Engraftment and development of human $CD34^+$-enriched cells from umbilical cord blood in NOD/LtSz-*scid/scid* mice. *Blood.* **90**:85–96.
7. Bensinger, W.I., et al. 1996. Transplantation of allogeneic $CD34^+$ peripheral blood stem cells in patients with advanced hematologic malignancy. *Blood.* **88**:4132–4138.
8. Dunbar, C.E., et al. 1995. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood.* **85**:3048–3057.
9. Negrin, R.S., et al. 2000. Transplantation of highly purified $CD34^+$ $Thy-1^+$ hematopoietic stem cells in patients with metastatic breast cancer. *Biol. Blood Marrow Transplant.* **6**:262–271.
10. Michallet, M., et al. 2000. Transplantation with selected autologous peripheral blood $CD34^+$ $Thy1^+$ hematopoietic stem cells (HSCs) in multiple myeloma: impact of HSC dose on engraftment, safety, and immune reconstitution. *Exp. Hematol.* **28**:858–870.
11. Terstappen, L.W.M.M., Huang, S., Safford, M., Lansdorp, P.M., and Loken, M.R. 1991. Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells. *Blood.* **77**:1218–1227.
12. Sutherland, H.J., Lansdorp, P.M., Henkelman, D.H., Eaves, A.C., and Eaves, C.J. 1990. Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc. Natl. Acad. Sci. USA.* **87**:3584–3588.
13. Bhatia, M., Bonnet, D., Murdoch, B., Gan, O.I., and Dick, J. 1998. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat. Med.* **4**:1038–1045.
14. Zanjani, E.D., Almeida-Porada, G., Livingston, A.G., Flake, A.W., and Ogawa, M. 1998. Human bone marrow $CD34^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to $CD34^+$ cells. *Exp. Hematol.* **26**:353–360.
15. Fujisaki, T., Berger, M.G., Rose-John, S., and Eaves, C.J. 1999. Rapid differentiation of a rare subset of adult human $lin^-CD34^-CD38^-$ cells stimulated by multiple growth factors in vitro. *Blood.* **94**:1926–1932.
16. Gallacher, L., et al. 2000. Isolation and characterization of human $CD34^-$ Lin^- and $CD34^+Lin^-$ hematopoietic stem cells using cell surface markers AC133 and CD7. *Blood.* **95**:2813–2820.
17. Nakamura, Y., et al. 1999. Ex vivo generation of $CD34^+$ cells from $CD34^-$ hematopoietic cells. *Blood.* **94**:4053–4059.
18. Yoder, M.C., et al. 1997. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity.* **7**:335–344.
19. Ito, T., Tajima, F., and Ogawa, M. 2000. Developmental changes of CD34 expression by murine hematopoietic stem cells. *Exp. Hematol.* **28**:1269–1273.
20. Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science.* **273**:242–245.
21. Sato, T., Laver, J.H., and Ogawa, M. 1999. Reversible expression of CD34 by murine hematopoietic stem cells. *Blood.* **94**:2548–2554.
22. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183**:1797–1806.
23. Yusa, K., and Tsuruo, T. 1989. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res.* **49**:5002–5006.

24. Spangrude, G.J., et al. 1991. Mouse hematopoietic stem cells. *Blood.* **78**:1395–1402.
25. Miller, C.L., and Eaves, C.J. 1997. Expansion *in vitro* of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proc. Natl. Acad. Sci. USA.* **94**:13648–13653.
26. Goodell, M.A., et al. 1997. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3**:1337–1345.
27. Storms, R.W., Goodell, M.A., Fisher, A., Mulligan, R.C., and Smith, C. 2000. Hoechst dye efflux reveals a novel $CD7^+CD34^-$ lymphoid progenitor in human unbilical cord blood. *Blood.* **96**:2125–2133.
28. Nishikawa, S.-I., et al. 1998. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity.* **8**:761–769.
29. Shultz, L.D., et al. 1995. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**:180–191.
30. Schmitt, C., Eaves, C.J., and Lansdorp, P.M. 1991. Expression of CD34 on human B cell precursors. *Clin. Exp. Immunol.* **85**:168–173.
31. Hogge, D.E., Lansdorp, P.M., Reid, D., Gerhard, B., and Eaves, C.J. 1996. Enhanced detection, maintenance and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human Steel factor, interleukin-3 and granulocyte colony-stimulating factor. *Blood.* **88**:3765–3773.
32. Unkeless, J.C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* **150**:580–596.
33. Conneally, E., Cashman, J., Petzer, A., and Eaves, C. 1997. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-*scid/scid* mice. *Proc. Natl. Acad. Sci. USA.* **94**:9836–9841.
34. Cashman, J., Bockhold, K., Hogge, D.E., Eaves, A.C., and Eaves, C.J. 1997. Sustained proliferation, multi-lineage differentiation and maintenance of primitive human haematopoietic cells in NOD/SCID mice transplanted with human cord blood. *Br. J. Haematol.* **97**:1026–1036.
35. Holyoake, T.L., Nicolini, F.E., and Eaves, C.J. 1999. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp. Hematol.* **27**:1418–1427.
36. Nicolini, F.E., et al. 1999. Unique differentiation programs of human fetal liver stem cells revealed both in vitro and in vivo in NOD/SCID mice. *Blood.* **94**:2686–2695.
37. Jackson, K.A., Mi, T., and Goodell, M.A. 1999. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc. Natl. Acad. Sci. USA.* **96**:14482–14486.
38. Zhou, S., et al. 2000. Expression of the breast cancer resistance protein (BCRP) in side population (SP) stem cells, natural killer (NK) lymphocytes, and erythroid progenitor cells. *Blood.* **96**:820a. (Abstr.)
39. Scharenberg, C.W., Harkey, M.A., and Torok-Storb, B.J. 2000. The transporter ABCG2 effluxes hoechst and its expression is restricted to the most primitive human hematopoietic cells. *Blood.* **96**:666a. (Abstr.)
40. Allikmets, R., Schriml, L.M., Hutchinson, A., Romano-Spica, V., and Dean, M. 1998. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res.* **58**:5337–5339.
41. Doyle, L.A., et al. 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. USA.* **95**:15665–15670.
42. Miyake, K., et al. 1999. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res.* **59**:8–13.
43. Scheffer, G.L., et al. 2000. Breast cancer resistance protein is localized at the plasma membrane in mitoxantrone- and topotecan-resistant cell lines. *Cancer Res.* **60**:2589–2593.
44. Rocchi, E., et al. 2000. The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. *Biochem. Biophys. Res. Commun.* **271**:42–46.
45. Hao, Q.L., Thiemann, F.T., Petersen, D., Smogorzewska, E.M., and Crooks, G.M. 1996. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood.* **88**:3306–3313.
46. Civin, C.I., et al. 1996. Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. *Blood.* **88**:4102–4109.
47. Oh, I.-H., Lau, A., and Eaves, C.J. 2000. During ontogeny primitive ($CD34^+CD38^-$) hematopoietic cells show altered expression of a subset of genes associated with early cytokine and differentiation responses of their adult counterparts. *Blood.* **96**:4160–4168.
48. Boggs, D.R. 1984. The total marrow mass of the mouse: a simplified method of measurement. *Am. J. Hematol.* **16**:277–286.