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

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Cells with a verapamil-sensitive ability to efflux Hoechst 33342 (termed side population [SP] cells) have been identified in adult marrow from several species including humans and in several tissues from adult mice. In mice, the SP phenotype appears to be a common feature of stem cells, but human SP cells have been less well characterized. We show here, for the first time to our knowledge, that SP cells are present in the second-trimester human fetal liver. They include all of the transplantable human hematopoietic stem cell activity detectable in NOD/SCID mice and also certain other, more differentiated hematopoietic cell types. Notably, the stem cell activity was confined to the CD34+CD38– SP+ population, and isolation of these cells gave an approximately tenfold enrichment of transplantable stem cells. This subset was not, however, coenriched in hematopoietic progenitors detectable by either short- or long-term in vitro assays, indicating most of these to be distinct from transplantable stem cells. These findings suggest that the SP phenotype is an important and distinguishing property of human hematopoietic stem cells and that early in ontogeny they express CD34.

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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.

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$^+$	CD34$^-$
	(23% ± 10% of all SP cells)	(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-den-
sity (<1.077 g/ml) cells were first isolated by centrifu-
gation on Ficoll-Hypaque (Pharmacia Biotech A/B,
Uppsala, Sweden). However, for later progenitor
assays, this first step was omitted, which enabled high-
er yields of SP cells to be obtained after the removal of
lin$^+$ cells (data not shown).

Animals. NOD/SCID mice (29) were bred and main-
tained 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 pas-
sage of the cells through the column were kept
overnight at 4°C in Iscove's medium supplemented
with a serum substitute (BIT 9500; StemCell Tech-
nologies) plus 40 µg/ml of human low-density
lipoprotein (Sigma Chemical Co.) and were then incu-
bated 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 pres-
ence of 50 µM verapamil (Sigma Chemical Co.). Cells
were then washed, resuspended in ice-cold HBSS con-
taining 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, Cana-
da; ref. 30), CD3 (Leu-4), CD7 (Leu-9), CD14 (MΦP9),
CD16 (NKP15), and CD66b (G10F5), and PE-conju-
gated 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 Dickin-
son 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 sort-
ed 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 Tech-
nologies), 50 ng/ml of recombinant human Steel fac-
tor (SF; prepared in the Terry Fox Laboratory), and 20
ng/ml each of human IL-3 (Novartis, Basel, Switzer-
land), human IL-6 (Cangene, Mississauga, Ontario,
Canada.), human G-CSF (StemCell Technologies), and
human GM-CSF (Novartis) to assess their direct gran-
ulopoietic, 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 (Bec-
ton Dickinson Immunocytometry Systems) or antihu-
man 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
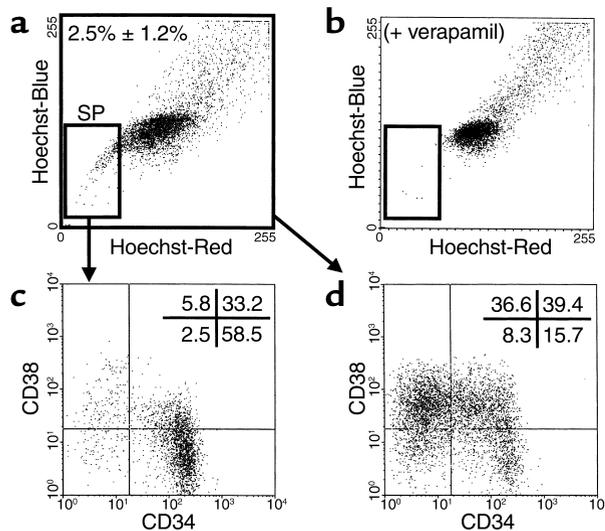
Figure 1

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 × 10³ to 285 ×10³ 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 associ-

ation 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.
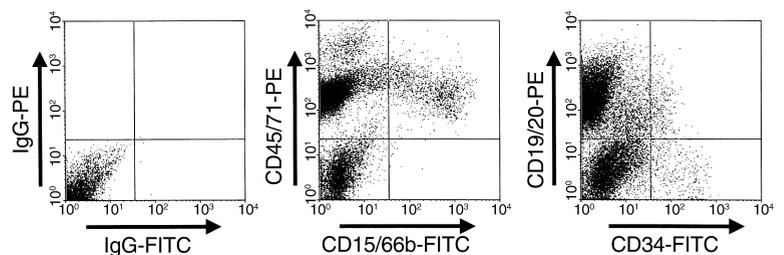
Figure 2
Representative FACS dot plot showing human multilineage engraftment in a secondary NOD/SCID recipient of initially transplanted SP fetal liver cells. The bone marrow cells were removed from both femurs and both tibias of a first NOD/SCID mouse transplanted 7 weeks previously with 22,000 SP CD34^{+}CD38^{-} 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

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