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Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor

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ABSTRACT **The maintenance and differentiation of hematopoietic stem cells is influenced by cells making up the hematopoietic microenvironment (HM), including bone marrow-derived stromal cells. We and several other investigators have recently demonstrated the molecular basis of abnormal HM observed in the steel mutant mouse and cloned the normal cDNA products of this gene (termed SCF, KL, or MCF). In this report, we focus on the human counterpart of the mouse Steel (*Sl*) gene. Alternative splicing of the human SCF pre-mRNA transcript results in secreted and membrane-bound forms of the protein. To investigate the role of these two forms of human SCF, we targeted an immortalized stromal cell line derived from fetal murine homozygous (*Sl/Sl*) SCF-deficient embryos for gene transfer of various human cDNAs encoding SCF. We report that stable stromal cell transfectants can differentially process the two forms of human SCF protein product. We also demonstrate that both soluble SCF and membrane-bound SCF are active in increasing the number of human progenitor cells in the context of stromal cell cultures, although in a qualitatively different manner. Hence, the membrane-bound form of SCF may play an important role in the cell–cell interactions observed between stromal and hematopoietic cells both *in vitro* and *in vivo*.**

The concept that the hematopoietic microenvironment (HM) plays an active role in hematopoiesis was postulated by Trentin, Wolf, and coworkers (1) based on observations indicating that direct cellular interactions between hematopoietic cells and stromal cells mediate cellular differentiation. Analysis of the HM has been greatly aided by the Steel (*Sl*) and dominant white spotting (*W*) mutations of the mouse, which display a common phenotype of sterility, impaired pigmentation, severe anemia, and other hematological defects (2). The *W* locus encodes the cellular homologue of the v-*kit* oncogene, the c-kit tyrosine kinase receptor (3, 4). Recently, the product of the *Sl* gene locus and its rat and human homologues has been cloned in a number of laboratories including ours (5–9). This gene product, termed SCF (stem cell factor), MGF (mast cell growth factor), KL (kit ligand), or steel factor (SF) encodes a stromal growth factor that exists in both membrane-bound and soluble forms. Most severe *Sl* mutations contain a deletion encompassing all (or most) of the *Sl* gene coding sequences, and mice homozygous for these mutations are not viable (5, 6).

The *Sl* gene encodes a primary translation product of 248 amino acids with a leader sequence and extracellular, transmembrane, and cytoplasmic domains. The resulting protein contains a proteolytic cleavage site encoded by exon 6 sequences (between amino acids 149 and 177) and posttranslational processing at this site leads to the secretion of a 165-amino acid biologically active protein (see Fig. 1) (10). An alternatively spliced cDNA form codes for a smaller 220-amino acid polypeptide that lacks exon 6 sequences including the proteolytic cleavage site and hence results in a membrane-bound protein (11) (K.M.Z., unpublished data). The viable *Sl*-Dickie(*Sl^d*) allele, which shows all of the pleiotropic effects seen in *Sl/Sl* mutants, encodes a smaller product of 183 amino acids due to a deletion of the genomic regions encoding the transmembrane and cytoplasmic domains (11, 12) (K.M.Z., unpublished results). Steel factor synergizes with lineage-specific growth factors to increase the size and number of murine and human progenitor hematopoietic colonies (13–16).

We have analyzed the role of the two forms of the normal *Sl* gene product within the context of the HM. For this purpose, we used a stromal cell line (Sl/Sl^4) derived from the HM of a homozygous (*Sl/Sl*) murine embryo, which has previously been shown to contain a deletion of the entire *Sl* gene coding sequence, for transfection and analysis of expression and activity of human (h) SCF cDNAs. We report stable transfectants of Sl/Sl^4, containing either the hSCF^{248} (including exon 6) cDNA or hSCF^{220} (lacking exon 6) cDNA were generated and the expression of the introduced cDNAs was analyzed. The transfected hSCF cDNAs are stably expressed and processed by murine stromal cells. Stromal cells expressing the soluble form of hSCF supported increased numbers of human hematopoietic progenitors in long-term marrow cultures (LTMC), in contrast to the Steel-deficient parental cell line. Comparable results were obtained when soluble recombinant hSCF was added to the cultures. Stromal cells expressing the membrane-bound form (hSCF^{220}) supported the maintenance of human progenitors in culture for up to 4 weeks even in the absence of demonstrable SCF activity in the culture supernatant, indicating that membrane-bound SCF may play an active role in the cellular interactions that occur in the HM.

MATERIALS AND METHODS

Generation and Analysis of Sl/Sl^4 Stable Transfectants. Alternatively spliced forms of hSCF cDNAs inserted into pJT-1(hSCF^{220}) (17) or pDSRα(hSCF^{248}) (17) vectors and expressed off the simian virus 40 early promoter were cotransfected with the p48 vector encoding hygromycin B (18) using the Lipofectin method as described by the manufacturer

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Abbreviations: HM, hematopoietic microenvironment; SCF, stem cell factor; hSCF, human SCF.

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(GIBCO/BRL). The following day cells were fed with Dulbecco's modified Eagle's medium with 20% calf serum (GIBCO) and hygromycin B (200 μg/ml) and resulting individual clones were picked, expanded, and analyzed for expression.

For protein analysis, stable transfectant cell lines were labeled overnight with [^{35}S]Met-Cys (ICN), supernatant and nonionic detergent lysates were prepared, and approximately equal portions were subjected to immunoprecipitation by the method of Usuki *et al.* (19) with rabbit antiserum generated against purified recombinant hSCF^{164} protein. Half of the immunoprecipitates were digested with neuraminidase, *O*-glycanase, and *N*-glycanase (glycosidase positive).

The stable transfectant cell lines were analyzed for membrane-bound hSCF by incubation of EDTA released cells with a monoclonal antibody (7H6) against hSCF^{164}, followed by labeling with goat anti-mouse immunoglobulin fluorescein isothiocyanate (Southern Biotechnology, Birmingham, AL) on a FACScan analytical flow cytometer with Consort 30 software for acquisition (Becton Dickinson). Subsequent list mode statistical analysis was performed on a HP 340 series workstation (Hewlett–Packard) with LYSYS II software (Becton Dickinson).

Conditioned media from stable transfectants were tested for the presence of secreted hSCF in a radioreceptor binding assay as follows: membrane preparations were made from human erythroleukemia cell line OCIM-1 (expressing 500,000 c-kit receptors per cell) (a gift from T. Papayannopoulou, University of Washington, Seattle) essentially as described (20). The membranes were incubated with ^{125}I-labeled hSCF produced in CHO cells and supernatants were used to compete for binding. Quantitation was accomplished by comparison to a purified standard hSCF preparation from CHO cells.

Establishment of Long-Term Cultures by Using Sl/Sl^4 Transfectant Cell Lines. Sl/Sl^4 clones were treated with mitomycin C (5 μg/ml) (Sigma) for 2 hr at 37°C, washed extensively with phosphate-buffered saline, treated with trypsin, and plated to confluence (3×10^5 cells per well) on six-well tissue culture plates (Falcon) pretreated with 0.1% gelatin (Sigma). The next day, human bone marrow was obtained with informed consent (according to protocol approved by the Human Investigation Committees of Children's Hospital and Indiana University) and mononuclear cells were isolated after separation on a Ficoll/Hypaque gradient (Pharmacia). Low density mononuclear cells were added ($1–5 \times 10^5$ cells per 35-mm well containing stromal cells) in Iscove's modified Dulbecco's medium (GIBCO) containing 10% fetal calf serum (GIBCO), 10% horse serum (Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml) (both GIBCO), 1 μM hydrocortisone (Upjohn), and sodium chloride (320 mosmol). Cultures were fed weekly by removal of 50% of the medium and cells with addition of fresh medium.

To enrich for CD-34$^+$ cells, adherence-depleted and low-density human bone marrow cells were incubated with mouse anti-human CD34 antibody HPCA (10 μg/ml) (Becton Dickinson) for 30 min at 4°C with gentle rocking. After centrifugation and washing, cells were resuspended in 0.5 ml of Hanks' balanced saline solution (GIBCO) in the presence of sheep anti-mouse IgG-coated magnetic beads (Dynabeads, Dynal, Oslo) at a 4:1 bead/cell ratio and were allowed to rest on ice for 30 min. Cells were then separated three or four times with a magnetic particle concentrator (Dynal). This routinely gave a 15- to 20-fold enrichment of progenitor cells. Cells were washed and added at $0.7–2 \times 10^4$ cells per 35-mm well.

Nonadherent cells collected at each weekly feeding were used for cytospin preparations and for plating in progenitor cultures. Progenitor cultures were completed as described (21) with 5 units of recombinant human erythropoietin per ml (Amgen), 100 ng of recombinant hSCF per ml (Amgen), and 70 ng of recombinant human interleukin 3 per ml (Genzyme). These cultures were incubated at 37°C in 5% CO_2/95% air for 14 days and colonies (>50 cells) were scored by morphology at day 14.

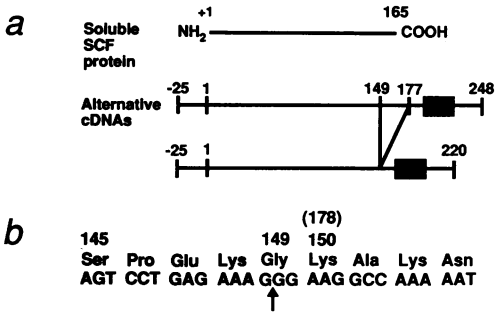

FIG. 1. cDNA structures and protein products of the Steel gene. (*a*) Schematic representation of the soluble form of hSCF protein and two alternative forms of hSCF cDNA. In the diagrams of the cDNA structures, numbers represent positions of amino acids and solid boxes indicate regions encoding the transmembrane domain. (*b*) Nucleotide sequence of the junction between exons 5 and 7 in the cDNA encoding SCF^{220}. Arrow indicates position of splice junction. Number in parentheses indicates amino acid position in hSCF^{248}.

RESULTS

Expression of hSCF^{220} and hSCF^{248} cDNAs in Stable Transfectants of Stromal Cell Line Sl/Sl^4. Sl/Sl^4 was cotransfected with a vector encoding hygromycin B phosphotransferase and either hSCF^{248} or hSCF^{220} cDNA (Fig. 1). Hygromycin-resistant clones, which contained SCF-hybridizing transcripts with or without exon 6 by RNA analysis, were further characterized for protein expression. Fig. 2 shows that Sl/Sl^4 hSCF^{248}-1 transfectant secretes ≈15 ng of hSCF per ml, while neither the parent cell line (Sl/Sl^4) nor transfectants containing the hSCF^{220} cDNA (such as Sl/Sl^4 hSCF^{220}-3) secrete detectable amounts of SCF. However, polyclonal antibody against hSCF reacts with protein in the cell lysates from the stable clones transfected with either the 220- or 248-amino acid cDNAs to yield unresolved, heterogeneously glycosylated forms (Fig. 3). After extensive deglycosylation, Sl/Sl^4 hSCF^{220}-3 and Sl/Sl^4 hSCF^{248}-1 each yields a single band of 22 and 30 kDa, respectively (Fig. 3*A*, arrows). In the supernatants, Sl/Sl^4 hSCF^{248}-1 produces two major glycosylated forms of 25 and 31 kDa (Fig. 3 *B* and *C*, arrows), which upon deglycosylation produces one band at 17 kDa (Fig. 3 *B* and *C*, asterisks). SCF protein is not detected in the supernatant of

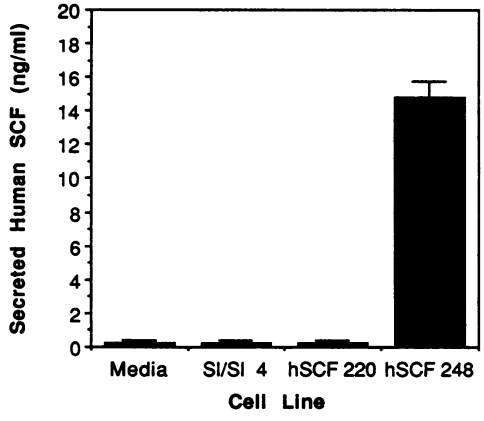

FIG. 2. Secretion of hSCF by transfected Sl/Sl^4 cells. Values are indicated for the medium control and supernatants from the following cell lines: parental Sl/Sl^4, Sl/Sl^4 hSCF^{220}-3 (hSCF220), and Sl/Sl^4 hSCF^{248}-1 (hSCF248). Bars represent mean ±SD.

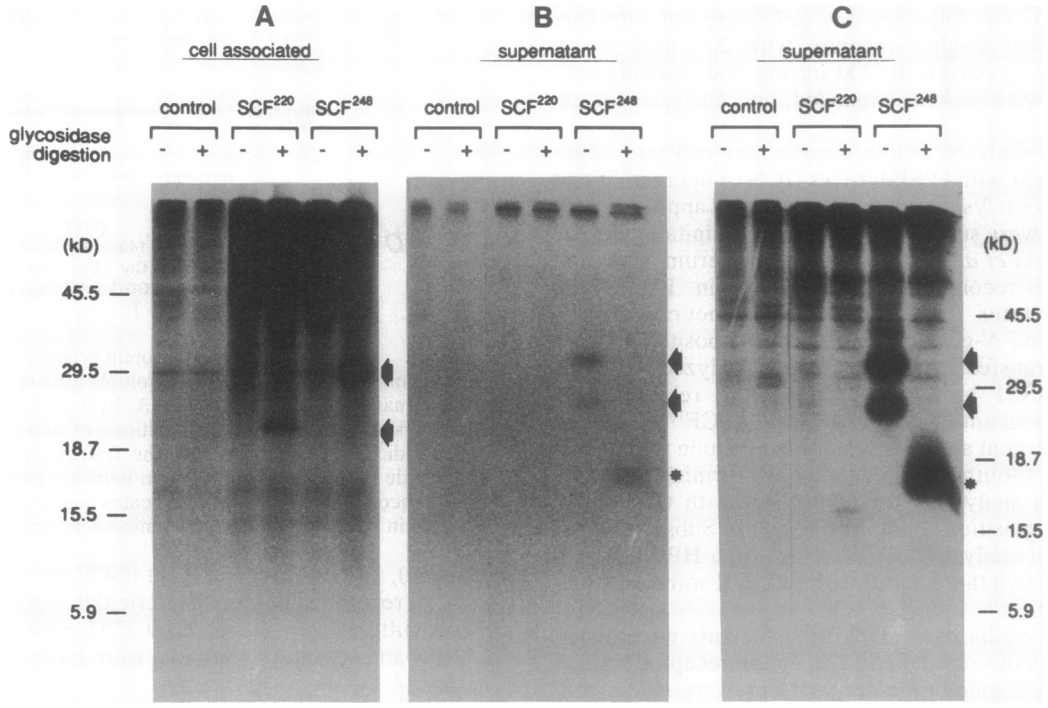

FIG. 3. Immunoprecipitation of hSCF protein from transfected Sl/Sl^4 cells. Cell lines described in the legend to Fig. 2 were used. Half of the immunoprecipitates were digested with neuraminidase, *O*-glycanase, and *N*-glycanase (glycosidase positive). (*A* and *B*) One-day exposures. (*C*) Ten-day exposure of the gel in *B*.

Sl/Sl^4 hSCF^{220}-3, except after very long exposure of the autoradiogram, when two faint bands are seen at 16 and 16.5 kDa, representing the deglycosylated forms (Fig. 3*C*).

To assay for the presence of hSCF protein on the cell surface of transfectants, analysis by fluorescence flow cytometry was performed. Compared to the parental Sl/Sl^4 cell line (Fig. 4*A*), the Sl/Sl^4 hSCF^{220}-3 transfectant demonstrates a high level of membrane-associated hSCF protein as measured by fluorescein isothiocyanate goat anti-mouse staining of cells labeled with a primary monoclonal antibody to hSCF (Fig. 4*B*), while Sl/Sl^4 hSCF^{248}-1 demonstrates a low level of membrane-associated SCF (Fig. 4*C*). Similar experiments were reported by Flanagan *et al.* (11) with murine Steel factor cDNAs transfected into COS-7 cells.

Support of Human Hematopoiesis by Murine Sl/Sl^4 hSCF Stromal Transfectants. To assess the biological significance of alternatively presented forms of the hSCF protein, the ability of genetically modified stromal cell lines to support human hematopoiesis *in vitro* was tested. Human bone marrow was added to each cell line and the cultures were incubated under standard conditions for LTMC. For up to 4 weeks in culture, the nonadherent cells present in Sl/Sl^4 hSCF^{220}-3 cultures consisted of mast cells, eosinophils, megakaryocytes, macrophages, granulocytes, nucleated erythrocytes, and blasts. In marked contrast, differentiation in standard LTMC is restricted almost entirely to macrophages and granulocytes.

The Sl/Sl^4 hSCF^{248}-1 cell line supported increased numbers of progenitors (compared to control) at weeks 2 and 3, demonstrating that the secreted SCF^{248} protein made by the transfected murine cell line is biologically active on human bone marrow cells. Table 1 shows the results of one representative experiment in which nonadherent cells from at least three wells were pooled and analyzed for each time point.

Table 1. Maintenance of human progenitor cells on hSCF cDNA transfected stromal cell lines

		Colonies per culture†			Colonies per culture‡		
	wk	CFU-GM	BFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-Mix
Sl/Sl^4	1	42	4	0	14 ± 4	4 ± 3	1 ± 1
	2	73 ± 9.8	39.3 ± 8.0	0.7 ± 0.6	0	0	0
	3	8.7 ± 2.0	8.3 ± 2.5	0.3 ± 0.6	—	—	—
	4	1.3 ± 1.1	1.7 ± 0.6	0.3 ± 0.6	—	—	—
Sl/Sl^4	1	37.3 ± 10.8	2.7 ± 0.6	0	395 ± 42*	80 ± 2*	15 ± 5*
hSCF^{248}-1	2	97.0 ± 13.5*	38.7 ± 1.6	2.0 ± 2.6	0	0	0
	3	23.3 ± 4.0*	6.3 ± 2.0	0	—	—	—
	4	1.3 ± 1.1	0.3 ± 0.6	0	—	—	—
Sl/Sl^4	1	31.0 ± 9.2	4.0 ± 2.6	0.7 ± 1.2	160 ± 2	18 ± 10	13 ± 4
hSCF^{220}-3	2	97.3 ± 3.0	37.0 ± 2.6	3.0 ± 3	151 ± 12**	2 ± 1**	0
	3	17.7 ± 7.3	4.7 ± 1.5	0	53 ± 4**	0	0
	4	23.7 ± 4.0**	4.0 ± 1.0**	0.7 ± 0.6	—	—	—

Means ± SD of triplicate cultures. *, $P < 0.05$ (hSCF^{248} vs. Sl^4 and hSCF^{220}); **, $P < 0.05$ (hSCF^{220} vs. hSCF^{248}) by Student's *t* test. CFU-GM, colony-forming units (granulocyte–macrophage); BFU-E, burst-forming units (erythroid); CFU-Mix, erythroid–myeloid mixed colonies.

†Whole bone marrow: 4×10^5 cells added per well.

‡CD34$^+$ bone marrow: 4×10^4 cells added per well.

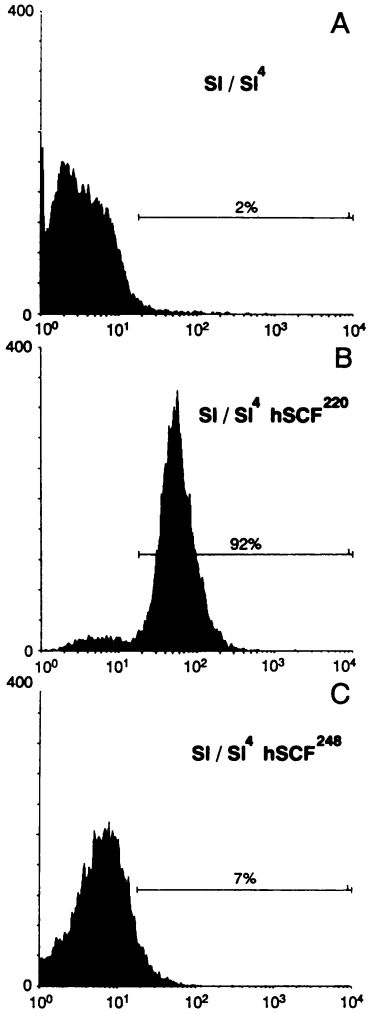

FIG. 4. Cell-associated hSCF in transfected Sl/Sl^4 cells. Cell lines described in Fig. 2 legend were analyzed as described in the text. (*A*) Sl/Sl^4 parent cell line. (*B*) Sl/Sl^4 hSCF^{220}-3 stable transfectant. (*C*) Sl/Sl^4 hSCF^{248}-1 stable transfectant. Percentages represent % cells judged to be expressing membrane-associated hSCF by fluorescence intensity.

Cultures on Sl/Sl^4 hSCF^{220}-3 were characterized by significant numbers of progenitors for up to 4 weeks in culture, at least 1–2 weeks longer than the Sl/Sl^4 hSCF^{248} supported cultures (a total of five independent experiments, one shown here). In the experiments represented in Table 1, several randomly picked myeloid colonies were noted to contain nucleated erythrocytes and megakaryocytes when examined microscopically. Therefore, the number of mixed progenitors is likely underrepresented in Tables 1 and 2.

The differences in cellularity and maintenance of progenitor populations by stable transfectant cell lines were also seen when CD-34$^+$-enriched bone marrow was used to initiate the long-term bone marrow cultures (Table 1). CD-34$^+$-enriched cells yielded cultures characterized by a transient burst of hematopoiesis when plated on the Sl/Sl^4 hSCF^{248}-1 cell line, while identical cell samples initiated sustained hematopoiesis when plated on Sl/Sl^4 hSCF^{220}-3 cell line (Table 1). In each experiment (a total of three independent experiments, one shown here), the duration of hematopoiesis was 1–2 weeks longer in cultures initiated on Sl/Sl^4 hSCF^{220}-3 cells than on either Sl/Sl^4 with addition of soluble SCF or on Sl/Sl^4 hSCF^{248}-1.

To demonstrate that the amount of SCF produced by Sl/Sl^4 hSCF^{248}-1 was not limiting the long-term maintenance of human hematopoiesis in this culture system, we added increasing amounts (1, 10, and 100 ng/ml) of soluble recombinant hSCF one or two times weekly to cultures of human bone marrow on the parental Sl/Sl^4 line (Table 2). The addition of 1–100 ng of soluble recombinant hSCF per ml resulted in an increase in progenitor numbers at weeks 1 and 2 when compared to Sl/Sl^4 cultures alone. There was no significant difference between the numbers of progenitors in cultures with 1, 10, or 100 ng of added SCF per ml, suggesting that even a low amount of soluble SCF is sufficient for this effect in the context of stromal-associated hematopoiesis. In no case did the addition of excess soluble factor increase the longevity of the cultures, a finding consistent with the results obtained in the Sl/Sl^4 hSCF^{248}-1 cultures. Larger numbers of progenitors are also seen in cultures on Sl/Sl^4 hSCF^{220}-3 when soluble factor was added but the longevity of the cultures was not affected (experiment done twice, results of one experiment shown here).

Table 2. Effect of excess soluble SCF on human progenitor maintenance on transfected stromal cell lines

	wk	Total colonies per culture†
Sl/Sl^4	1	113 ± 30
	2	43 ± 8
	3	3 ± 2
Sl/Sl^4	1	293 ± 80*
+ 1 ng/ml‡	2	103 ± 30*
	3	1 ± 2
Sl/Sl^4	1	330 ± 5*
+ 10 ng/ml‡	2	170 ± 8*
	3	5 ± 2
Sl/Sl^4	1	387 ± 55*
+ 100 ng/ml‡	2	120 ± 17*
	3	2 ± 1
Sl/Sl^4	1	277 ± 60
hSCF^{220}-3	2	190 ± 35**
	3	63 ± 3**
Sl/Sl^4	1	395 ± 10
hSCF^{220}-3	2	210 ± 32
+ 100 ng/ml‡	3	75 ± 11

*, NS (1 ng vs. 100 ng of SCF added), $P < 0.05$ (SCF added vs. Sl^4); **, $P < 0.05$ (hSCF^{220} vs. Sl^4, hSCF^{220} vs. Sl^4 + SCF) by Student's *t* test.
†CD34$^+$-enriched cells were added (1.5 × 10^4 cells per culture).
‡Amount of SCF added.

DISCUSSION

Hematopoiesis occurs both *in vivo* and *in vitro* in a complex HM made up of multiple adherent cell types (1, 22). The gene defective in the murine Steel mutation, which has previously been demonstrated to be a defect in this HM, has recently been cloned and the protein product was expressed (5–9). The presence of alternative-spliced RNA transcriptional products and posttranslational processing generates both secreted and membrane-bound forms of this protein, although the physiological relevance of these forms has been unclear. Since the molecular lesion associated with the viable *Sl^d* allele has been shown to be a deletion of the transmembrane domain of the Steel protein (11, 12) (K.M.Z., unpublished results), the presence of a defective hematopoietic phenotype in this mouse implicates the membrane-bound form of Steel factor as an important component of the HM.

We have sought to study the role of the alternatively spliced hSCF products in a physiologically relevant hematopoietic culture system. For this purpose, stromal cell lines cultured from murine fetal liver during the developmental stage at which this organ is the major site of hematopoiesis represent ideal cells for the introduction of SCF cDNAs. This

system may offer advantages over other cells such as COS-7 cells (monkey kidney cells) (11), which may not possess the same proteases or secretory pathways as HM cells and which show transient expression in only a fraction of cells. Furthermore, since the action of SCF on primitive hematopoietic populations occurs in synergy with other growth factors, stromal cell lines offer the advantage that growth factors normally synthesized by the HM are present in the appropriate cellular context.

Regulation of SCF expression by stromal cells could occur both at the level of alternative splicing of pre-mRNA to include or exclude exon 6 and at the level of posttranslational processing. In these experiments, we have demonstrated that murine stromal cells derived from an appropriate HM process the alternative hSCF proteins with a high degree of discrimination. The 248-amino acid form is efficiently cleaved by a stromal cell protease to yield biologically active, secreted hSCF, while the 220-amino acid form is exclusively retained on the cell surface.

Experiments presented here, with either whole or CD34-enriched normal bone marrow from different donors, demonstrate that significant numbers of human progenitor cells persist in cultures established on murine stromal cells expressing either the $hSCF^{220}$ or the $hSCF^{248}$ cDNA. These colonies consisted of erythroid and myeloid as well as myeloid–megakaryocytic and some erythroid–myeloid mixed colonies. Subsequent weeks in culture resulted in a reduction of Sl/Sl^4 $hSCF^{248}$-supported colony numbers, while cultures on Sl/Sl^4 $hSCF^{220}$-3 demonstrated significant numbers of human progenitors for up to 4 weeks. Hence, both the $hSCF^{248}$ and $hSCF^{220}$ transfectants show biological activity when assayed on human hematopoietic cells. Our data also suggest that hematopoiesis is supported longer *in vitro* in cultures established with membrane-bound SCF expressed in the stromal layer.

Detectable levels of secreted SCF from normal stromal cells are well under 1 ng/ml (K.M.Z., unpublished results). Therefore, the 15 ng of SCF per ml detected in the conditioned media from Sl/Sl^4 $hSCF^{248}$-1 transfectant represents a high level of protein expression. However, to assess whether the observed results were due to a suboptimal amount of SCF in the cultures, we included additional amounts of soluble recombinant hSCF in the cultures. Some increases in colony numbers (even at very low concentrations of soluble factor) were noted over weeks 1 and 2, but the length of time progenitor cells were detected in these cultures did not increase. This result emphasizes the difference between short-term liquid cultures (in which 50–100 ng of SCF per ml are optimal concentrations for progenitor growth) and stromal-associated culture in which even small amounts of SCF stimulate progenitor growth. Cultures of Sl/Sl^4 $hSCF^{220}$-3 transfectants expressing the membrane-bound form of SCF showed significant, albeit decreasing, numbers of progenitors at 3 and in most cases 4 weeks. The demonstration of activity of SCF^{220} cDNA on human hematopoietic progenitor cells in the context of stromal cells and the differences noted in these cultures compared to Sl/Sl^4 $hSCF^{248}$ cultures indicate that the membrane-bound form of SCF may play an important role in the cellular interactions that are known to occur in the HM *in vivo*.

Differences in hematopoietic cell response to soluble vs. membrane-bound growth factor could occur through several mechanisms, including changes in c-kit receptor internalization (23), generation of secondary signals in stromal cells via the intracytoplasmic domain of membrane-bound Steel factor, or increased colocalization of primitive cells to a "stem cell niche" (24) via adhesive properties of membrane-bound Steel factor, which may provide additional signals to the hematopoietic cell in a local area network. Further studies will be needed to ascertain both the exact role of Steel factor presentation in normal hematopoiesis and the mechanism(s) involved in these roles.

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