Soluble Stem Cell Factor in Human Serum

By Keith E. Langley, Larry G. Bennett, Jette Wyppych, Susan A. Yancik, Xiao-Dong Liu, Keith R. Westcott, David G. Chang, Kent A. Smith, and Krisztina M. Zsebo

Stem cell factor (SCF) is a recently described factor active in the early stages of hematopoiesis. It can exist in membrane-bound form and in proteolytically released soluble form. The levels and nature of SCF in human serum are described. As determined by an enzyme-linked immunosorbent assay performed for 257 samples, SCF level in serum averaged 3.3 ± 1.1 ng/mL. The serum SCF was partially purified by immunoaffinity chromatography and analyzed by glycosidase treatments in conjunction with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The results show that the SCF has N-linked and O-linked carbohydrate and corresponds to the soluble form, at or about 185 amino acids in length. The findings suggest functional importance for soluble SCF in humans. © 1993 by The American Society of Hematology.

Cytokines involved in hematopoiesis circulate in human serum and studies correlating these levels to various disease states have shed light on the roles for these molecules in normal host defense and in response to hematopoietic insult. We report here that stem cell factor (SCF) is present in human serum at levels readily detectable by immunosassay in the nanogram per milliliter range; and that partially purified SCF from human serum appears to be the soluble 165 amino acid form. The level of SCF found in serum gives substantial biologic responses in vitro.

In the mouse, SCF is the product of the steel (Sl) locus and the receptor for SCF, c-kit, is the product of the dominant white spotting (Ws) locus. Hematopoiesis, gametogenesis, melanogenesis (pigmentation), and mast cell development are affected by defects at Sl and Ws. The genes for rat, mouse, and human SCF have been cloned. The factor has also been referred to as mast cell growth factor, kit ligand, and steel factor. In hematopoiesis, the factor is early acting and affects multiple lines.

Mouse and human SCF mRNA undergo alternative splicing events that either include exon 6 (form 1) or exclude exon 6 (form 2) in the mature mRNA. The form 1 mRNA encodes a 25-amino acid signal sequence followed by 248 amino acids; of these 248 amino acids, 1 through 189 constitute the SCF extracellular domain, 190 through 212 represent a consensus hydrophobic transmembrane domain, and 213 through 248 constitute the intra-cytoplasmic region. The originally isolated natural rat SCF was comprised of amino acids 1 through 165. Thus, it appeared that SCF is initially synthesized as a membrane-bound polypeptide, and that proteolytic cleavage can subsequently lead to release of a soluble protein. The form 2 mRNA encodes a polypeptide of 220 amino acids plus the signal sequence. As a result of the exclusion of exon 6, a glycine is present in place of amino acids 149 through 177 of form 1. Transfections of the mouse form 1 SCF cDNA into COS-1 cells, or the human form 1 cDNA into Sl/SI stromal cells (lacking the genomic SCF gene) and into Chinese hamster ovary (CHO) cells results in a secreted SCF. The secreted human SCF from transfected CHO cells has been purified and shown to contain amino acids 1 through 165. However, transfection of the corresponding form 2 SCF cDNAs into all three cell lines results in a membrane-bound SCF polypeptide, probably because the proteolytic cleavage site encoded in exon 6 is lacking.

The relative occurrence and comparative roles of membrane-bound and soluble forms of SCF in vivo remain to be determined. Naturally occurring human SCF has not yet been isolated, and SCF processing in a completely human system has not been investigated. Therefore, we used an enzyme-linked immunosorbent assay (ELISA) to measure SCF levels in human serum, and we have studied the biochemical properties of the SCF that is found to be present.

Materials and Methods

ELISA for SCF. A sandwich-type ELISA was used. Anti-SCF polyclonal antibody was produced in New Zealand White rabbits by immunization with CHO cell-derived human SCF-164.21 SCF-specific antibody was affinity purified by binding to and elution from Actigel-ALD (Sterogene, Bioseparations, Inc, Arcadia, CA) results covalently attached CHO cell-derived human SCF-164.21 Anti-SCF monoclonal antibody (MoAb)-secreting hybridoma 7H6 was obtained after immunization of Balb/c mice with Escherichia coli-derived human SCF-164.21 IgG was purified from mouse ascites fluid using Protein G-Sepharose resin (Pharmacia LKB, Piscataway, NJ). Immunol 4 Removalwell Strips (Dynatech Laboratories, Inc, Chantilly, VA) were coated with the affinity-purified polyclonal antibody (24-hour incubation with 100 μL of antibody at 2.5 μg/mL in TEN buffer [50 mmol/L Tris-HCl, 10 mmol/L EDTA, 150 mmol/L NaCl, pH 7.4]) containing 0.02% Thimerosal [Sigma Chemical Co, St Louis, MO]) followed by blocking (further 24-hour incubation after addition of 200 μL of 1% bovine serum albumin, 5% sucrose in TEN buffer) and air drying. To carry out the ELISA, 50 μL 0.1% Tween 20 in TEN was added to each well, followed by 50 μL sample and incubation was performed for 4 hours at room temperature. The wells were washed twice with 0.01% Tween 20 in TEN buffer. Then 100 μL of 10% fetal bovine serum (FBS) in TEN buffer containing 0.5 μg/mL of purified 7H6 MoAb that had been conjugated to horseradish peroxidase (HRPO) was added. After 1 hour at room temperature, wells were washed four times with 0.1% Tween 20 in TEN buffer, and 100 μL TMB Microwell Peroxidase Substrate solution (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD) was added for HRPO reaction. Reactions were stopped with 100 μL 0.5 N H₂SO₄ after 30 minutes, and absorbances at 450 nm were read. Purified CHO cell-derived human SCF-165 diluted into human serum that had been depleted of SCF by passage over an anti-SCF immunoaffinity column (see Fig 2; immobilized 7H6 MoAb was used), served as standard. Standard curves were linear over the range 0.5 to 10 ng/mL. Coef-

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Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed according to Laemmli22; running gels contained 12.5% (wt/vol) acrylamide. Immunoblotting was performed according to Burnette23; immunocomplexes were visualized with a Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA).

Assay for peripheral blood erythroid colony-forming cells (burst-forming unit-erythroid [BFU-E]). Normal human peripheral blood leukocytes were fractionated by centrifugation using Leucoprep tubes (Becton Dickinson, Mountain View, CA), and low-density cells were used. Culturing in agar was performed as described for human marrow cells11 with erythropoietin present at 50 U/mL, peripheral blood low-density cells present at 105 cells/mL, and various levels of CHO cell-derived human SCF115-20 or sample. After 14 days of incubation, colonies were counted using a dissecting microscope; all colonies were large hemoglobinized erythroid colonies.

RESULTS AND DISCUSSION

Serum samples from a total of 257 healthy volunteer blood donors were assayed in the ELISA. The data obtained are summarized in Fig 1. The average serum SCF level for this group is 3.31 ng/mL with a standard deviation of 1.09 ng/mL. Levels were fairly uniform among the samples tested.

SCF levels analyzed by sex of the serum donor are given in Table 1, part A. The samples were from approximately equal numbers of males and females; 125 were from males, 132 from females. The distribution of SCF levels was normal in both groups, but the variance was found to be statistically significantly different. Therefore, the Wilcoxon rank serum test was used to compare the groups, and the difference was found to be borderline significant (P value = .07). SCF levels were also analyzed by age group, as summarized in Table 1, part B. Levels for the four age groups were compared using the analysis of variance (ANOVA) method, and statistically significant difference was not obtained (P = .34). Levels can also be measured in various disease states or during bone marrow recovery after myelosuppressive therapy, and any differences could have diagnostic utility. It is possible that soluble c-kit receptor or other SCF binding proteins could be present in serum; however, the inclusion of a purified, recombinant-derived soluble version of the extracellular portion of the human c-kit receptor did not affect SCF quantitation by the ELISA (data not shown).

To characterize the molecular species of SCF present in serum, the SCF was partially purified by immunoadfinity chromatography. A purification summary is given in Table 2. The SCF in the column eluate appears to be about 4,500-

Fig 1. SCF concentrations in normal human serum samples, measured by ELISA. Each bar represents the number of samples falling between the values on the x-axis on either side of the bar. N = 257; mean = 3.31 ng/mL; standard deviation = 1.09 ng/mL; range = 1.31 to 7.96 ng/mL.

Table 1. SCF Concentrations in Normal Human Serum Samples, Measured by ELISA, Analyzed By Sex and Age

<table>
<thead>
<tr>
<th></th>
<th>No. of Samples</th>
<th>SCF Level (ng/mL)*</th>
</tr>
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<tbody>
<tr>
<td><strong>Total</strong></td>
<td>257</td>
<td>3.31 ± 1.09</td>
</tr>
<tr>
<td><strong>A. Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>125</td>
<td>3.46 ± 1.22</td>
</tr>
<tr>
<td>Female</td>
<td>132</td>
<td>3.17 ± 0.97</td>
</tr>
<tr>
<td><strong>B. Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-45 yrs</td>
<td>63</td>
<td>3.28 ± 1.13</td>
</tr>
<tr>
<td>&gt;50 yrs</td>
<td>44</td>
<td>3.26 ± 1.16</td>
</tr>
</tbody>
</table>

* Means ± SDs are given.

Glycosidase treatments with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Aliquots (24 μL) of the concentrated eluate from immunoadfinity chromatography, and aliquots of CHO cell-derived human SCF115-20 were treated with glycosidases at 37°C for 22 hours in a total volume of 60 μL that included 5 mmol/L 3-(3-cholamidopropyl)dimethylammonio)-1-propane-sulfonate (CHAPS), 0.02% sodium azide, and 33 mmol/L 2-mercaptoethanol. Enzymes used were neuraminidase (from *Arthrobacter ureaficiens*; Calbiochem; 0.5 U/mL), O-glycanase (Genzyme; endo-α-N-acetyl-galactosaminidase; 15 μmol/mL), and N-glycanase (Genzyme; peptide: N-glycosidase F; peptide: N*-N-acetyl-β-glucosaminyl-[asparagine amidase; 10 U/mL]. SDS-PAGE was performed according to Laemmli22; running gels contained 12.5% (wt/vol) acrylamide. Immunoblotting was performed according to Burnette23; immunocomplexes were visualized with a Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA).

Immunoaffinity chromatography. For anti-SCF immunoaffinity chromatography, 775 mg of rabbit anti-SCF polyclonal antibody (see legend of Fig 1), puritshed with Affigel Protein A (Bio-Rad Laboratories, Richmond, CA) was coupled to 155 mL of swelled CNBr-activated Sepharose 4B resin (Pharmacia) with subsequent blocking and washing according to the manufacturer's instructions. A column (4.4 × 10.2 cm), equilibrated with phosphate-buffered saline (PBS), was prepared. Twenty-one liters of pooled human serum (Biosell Laboratories, Inc; Rancho Dominguez, CA) was clarified by filtration, protease inhibitors were added (phenylmethylsulfonyl fluoride [PMSF], 2 mmol/L; leupeptin, 2 μg/mL; pepstatin, 1.4 μg/mL; EDTA, 9.5 mmol/L; benzamidine, 1.9 mmol/L), and the mixture was passed over the column at 920 mL/h at 4°C. After washing the column with 16,000 mL of PBS with extra NaCl of 350 mmol/L included, elution was performed using 100 mmol/L acetic acid, 300 mmol/L NaCl, pH 2.67. An eluate pool of 710 mL was collected, neutralized, and concentrated 30-fold.

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Table 2. Partial Purification of SCF From Human Serum Using Immunoaffinity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (L)</th>
<th>Total Protein (µg)*</th>
<th>Total SCF (µg)/f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>21</td>
<td>914</td>
<td>65</td>
</tr>
<tr>
<td>Immunoaffinity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>column run-through</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus wash</td>
<td>38</td>
<td>980</td>
<td>0</td>
</tr>
<tr>
<td>Immunoaffinity column eluate, concentrated†</td>
<td>0.024</td>
<td>0.084</td>
<td>27</td>
</tr>
</tbody>
</table>

* Measured by the method of Bradford4 using bovine serum albumin as standard.
† Measured by ELISA described in Materials and Methods.
‡ Eluate of 0.71 L concentrated using an Amicon stirred cell with YM10 membrane.

fold purified but is still only about 0.03% pure, ie, a further 3,100-fold purification would be required for homogeneity.

The degree of purification of SCF achieved by the immunoaffinity chromatography made it possible to detect the SCF by SDS-PAGE with immunoblotting using anti-SCF antibody. These analyses were done in conjunction with glycosidase treatments. The findings, in comparison with those for pure CHO cell-derived human SCF13-165, are shown in Fig 2. The results are like those reported for soluble natural rat SCF12,13 and for soluble CHO cell-derived rat and human SCFs.20,25 The CHO cell-derived human SCF13-165 migrates at about 18,500 daltons on gels when fully deglycosylated (comigrating with E coli-derived SCF13-165 25), and has been shown by precise structural analysis to terminate at amino acid 165.20 Thus, it is apparent from Fig 2 that human serum contains the soluble form of SCF, at or about 165 amino acids in length, with both N-linked and O-linked carbohydrate present. From the ELISA values of Table 2, the amount of partially purified SCF loaded in the gel lanes 5 through 8 of Fig 2A would be about 27 ng and would stain somewhat more lightly in the immunoblot than the 62.5 ng of CHO cell-derived SCF standard loaded in lanes 1 through 4. This does seem to be the case, and the comparison provides qualitative support for the validity of the serum SCF concentration values determined by ELISA.

Figure 3 shows an example of the peripheral blood erythroid colony forming cell assay performed with low-density cells from an individual human donor in response to recombinant glycosylated human SCF. In the case shown, the concentration of recombinant glycosylated SCF required for half-maximal effect is ~160 pmol/L (3 ng/mL; the SCF concentration values are expressed in terms of SCF monomer, using a molecular weight of 18,500 for the protein portion of each monomer making up the SCF dimer25). More generally, the concentrations of the recombinant glycosylated SCF required for half-maximal effect with cells from other individual donors have ranged from 2 to 9 ng/mL. Because

![Fig 2. SDS-PAGE and immunoblot analysis, with and without prior glycosidase treatments, of SCF purified from human serum using immunoaffinity. (A) Immunoblotting with rabbit polyclonal antibody raised against CHO cell-derived human SCF13-165 (dilution 1:500); (B) immunoblotting as in (A), but with pre-immune serum. Lanes 1 through 4, CHO cell-derived human SCF13-165 (0.0625 µg; reference 20); lanes 5 through 8, concentrated eluate from anti-SCF immunoaffinity chromatography of human serum (gel loads equivalent to 21 mL of human serum). Treatments before electrophoresis were as follows: lanes 1 and 5, no treatment; lanes 2 and 6, incubation only (without glycosidases); lanes 3 and 7, N-glycanase; lanes 4 and 8, neuraminidase, O-glycanase, and N-glycanase. The marks at the side of each gel indicate migration positions of standard proteins having molecular weights of 102 times the indicated numbers.](http://www.bloodjournal.org/article-pdf/...
tyrosine kinase receptors, both exist as dimers, both can be produced in membrane-bound or soluble forms, and the genetic differences between the serum-derived components which are inhibitory in the progenitor assay; there could be differences in carbohydrate structure) that manifest them-
selfs differently in the two assays; or the immunoaffinity purification procedure, which includes exposure to low pH, could be more detrimental to the progenitor cell activity than to the detection by ELISA.

SCF shares certain genetic and structural homologies with macrophage colony-stimulating factor (M-CSF); both bind tyrosine kinase receptors, both exist as dimers, both can be produced in membrane-bound or soluble forms, and the genomic exons for both correlate closely. High circulating level is yet another similarity, because M-CSF is present at about 6 to 8 ng/mL (~350 pmol/L) in normal human serum. In contrast, erythropoietin circulates in the 1 to 5 pmol/L range, has a kd in the 100 pmol/L range for receptor binding, and elicits half-maximal biologic activity with receptor occupancy of ~5%. Other CSFs such as granulocyte CSF (G-CSF) are present at very low levels in serum in the absence of infection. It is likely that both soluble and membrane-bound forms of SCF have roles in vivo. The alternative splicing of exon 6, which encodes the site of the proteolytic cleavage that generates the soluble form, could be involved in the regulation of soluble versus membrane-bound forms. Several lines of evidence suggest functional importance of membrane-bound SCF, and the present results characterizing SCF in human serum suggest that proteolytic release of soluble SCF occurs in vivo, and indicate functional importance for soluble SCF as well.

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**Fig 3.** Response of peripheral blood cells to SCF in vitro. The assay was performed as described in Materials and Methods, with the indicated concentrations of CHO cell-derived human SCF. Data are plotted as percent maximum colony number, which was 30 colonies per 2 x 10^5 low-density peripheral blood cells plated.


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