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Stem cell growth factor: *in situ* hybridization analysis on the gene expression, molecular characterization and *in vitro* proliferative activity of a recombinant preparation on primitive hematopoietic progenitor cells

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Introduction: *In situ* hybridization of whole mouse fetuses and their tibias with a stem cell growth factor (SCGF) antisense probe demonstrated specific expression of SCGF mRNA around skeletal tissues, particularly in bone marrow cells, proliferating chondrocytes, the perichondrium and periosteum, but little expression in resting or hypertrophic chondrocytes.
Methods: Recombinant human (rh) SCGF-α was purified from a conditioned medium of SCGF-α gene-transfected CHO cells. The molecular mass of rhSCGF-α, 45 kDa, was shifted down to 40 kDa by digestion with endo-O-glycosidase and sialidase, suggesting O-glycosylation of rhSCGF-α with sialic acids.
Results: For human bone marrow $CD34^+Lin^-$ cells, rhSCGF-α alone did not stimulate colony-formation, but small cluster-formation ($10.3 \pm 2.5/1 \times 10^3$ $CD34^+Lin^-$ cells). It promoted growth of erythroid and granulocyte/macrophage (GM) colonies in the primary culture with erythropoietin and GM colony-stimulating factor (CSF) or G-CSF, respectively, and further supported GM progenitor cells in a short-term liquid culture. In contrast, rhSCGF-α suppressed stem cell factor (SCF)-stimulated erythroid bursts, indicating some competitive interaction between SCGF and SCF. rhSCGF-α was synergistic with interleukin-3 and the *flt3* ligand to enhance GM colony-growth, but not synergistic with those inducing *ex vivo* expansion of GM progenitor cells.
Conclusion: SCGF is selectively produced by osseous and hematopoietic stromal cells, and can mediate their proliferative activity on primitive hematopoietic progenitor cells.
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Keywords: stem cell growth factor; *in situ* hybridization; recombinant preparation; hematopoiesis; stem cell factor; *flt*3 ligand

Introduction

Early primitive hematopoiesis is stimulated in concert by multiple growth factors, of which stem cell factor (SCF) and the *flt3* ligand (FL) are crucial.[1,2] We have previously demonstrated and tried to partially purify another growth factor for primitive hematopoietic progenitor cells, termed stem cell growth factor (SCGF), in a mitogen-stimulated leukocyte conditioned medium.[3] SCGF is demonstrated in the culture supernatants of KPB-M15 cells, a human myeloid cell line established from a patient with chronic myeloid leukemia in blast crisis.[4] SCGF alone does not exhibit colony-stimulating activity, but does have burst-promoting activity (BPA) and granulocyte/macrophage (GM) colony-promoting activity (GPA) on erythroid and GM progenitor cells (BFU-E and CFU-GM) in a primary semisolid clonal culture with erythropoietin (Epo) and GM colony-stimulating factor (CSF), respectively, and further CFU-GM-supporting activity during short-term liquid culture of bone marrow (BM) cells.

SCGF cDNA has been recently cloned from the cDNA library prepared from KBP-M15 cells, and characterized as a 1196 bp open reading frame encoding a novel 245 aa protein without a putative N-linked glycosylation site.[5] cDNA for mouse, rat and human full-length SCGF has been further isolated from their respective hematopoietic stromal cells, using

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a reverse transcriptase-polymerase chain reaction with the original SCGF oligonucleotide primers.[6] Newly cloned human full-length SCGF is a 323 aa protein. Therefore, we term it SCGF-α and the original 78 aa-deleted shorter clone as SCGF-β. SCGF has a conserved carbohydrate-recognition domain motif sequence at the COOH-terminal, suggesting that it is a new member of the C-type lectin superfamily. The human SCGF gene is located on chromosome 19 at position q13.3 and the mouse SCGF gene on chromosome 7 at syntenic position B3-B5, where genes for early-acting hematopoietic growth factors of FL and interleukin (IL)-11 are clustered. Previous studies showed that SCGF mRNA is highly expressed in hematopoietic stromal cells,[5,6] indicating that SCGF could be a mediator between primitive hematopoietic progenitor and stromal cells within the hematopoietic microenvironment.

In the present study, we investigated tissue expression of SCGF mRNA by *in situ* hybridization, prepared and characterized pure recombinant human (rh) SCGF-α from SCGF gene-transfected CHO cells, and evaluated the detailed *in vitro* activity of rhSCGF-α on human primitive hematopoietic progenitor cells, in conjunction with other growth factors.

Materials and methods

Tissue preparation

Fetuses were obtained from pregnant Crj:CD-1 (ICR) mice (18-day postcoitum, Charles River Japan, Yokohama, Japan). Whole fetuses and their tibias were fixed overnight with 10% formalin in phosphate-buffered saline, pH 7.4 (PBS). Serial 6 μm longitudinal paraffin sections were mounted on Superfrost/Plus precoated slides (Fischer Scientific, Pittsburgh, PA, USA).

Riboprobes

^{35}S-labeled riboprobes were prepared using mouse SCGF cDNA.[6] Antisense and sense [^{35}S]cRNAs were synthesized from linearized plasmids in the presence of [^{35}S]UTP (1289 Ci/nM; New England Nuclear Corp., Boston, MA, USA).

In situ *hybridization*

Sections were postfixed with 4% paraformaldehyde, digested with 1 μg/ml proteinase-K (Sigma, St. Louis, MO, USA), and acetylated with 0.25% acetic anhydride in the presence of 1 M triethanolamine. Hybridization was performed[7] with SCGF [^{35}S]cRNAs (5×10^7 c.p.m./ml) at 55°C for 18 h. Posthybridization washes were carried out at a final stringency of 55°C in 50% formamide and 2 × SSC, and unbound riboprobes were degraded with 10 μg/ml ribonuclease A (Nacalai tesque, Kyoto, Japan). Slides were dipped into NTB-2 (Eastman Kodak, New Haven, CT, USA) and stored at 4°C for up to four weeks. They were then developed, counterstained with hematoxylin and eosin, and mounted.

Monoclonal antibodies

Rats were immunized with a synthetic peptide Ac-REWEGGWGGAQEEEREREALC-OH that corresponded to amino acids 27–46 of the SCGF protein. Hybridoma were obtained according to the standard procedure.[8] The monoclonal antibody KM2142 was biotinylated[9] with ImmunoPure NHS-LC-Biotin (Pierce, Rockford, IL, USA). The biotinylated antibody was dialyzed against PBS to remove any unbound biotin.

Plasmid construction and expression of rhSCGF-α

Human SCGF-α cDNA[6] was subcloned into an animal cell expression vector pAGE210[10] to create pAGE-SCGF-α. The vector pAGE210 was a derivative of pAGE248;[11] the Moloney murine leukemia virus promoter (*Xho*I–*Hind*III fragment) of pAGE248 was replaced with the SV40 early promoter (*Xho*I–*Hind*III fragment) of pAGE103.[12] It possessed a hygromycin resistance gene able to select stable transformants and a dihydrofolate reductase gene for gene amplification by methotrexate (MTX) selection. Dihydrofolate reductase-deficient CHO cells were electroporated with pAGE-SCGF-α using a Bio-Rad Gene Pulser. Stably transformed cells were selected in an α-modification of Eagle's minimum essential medium (α-MEM, GIBCO/BRL, Rockville, MD, USA) containing 5% fetal calf serum (FCS, GIBCO/BRL, Auckland, New Zealand) and 300 μg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan). The SCGF-α gene was amplified with serially increasing concentrations of MTX (amethopterin, Sigma) in a nucleoside-free α-MEM with 5% FCS and hygromycin B.

Purification of rhSCGF-α

pAGE-SCGF-α-transfected CHO cells resistant to 200 nM MTX were cultivated in a roller bottle containing 200 ml of EX-CELL301 medium (JRH Bioscience, Lenexa, KS, USA) supplemented with 20 mM HEPES, pH 7.6. The CHO cell-culture supernatant was applied to a Zn^{2+} chelating-Sepharose FF (15/10) column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate buffer, pH 7.2 containing 0.5 M NaCl. Fractions eluted with a linear gradient of 0–100 mM histidine were precipitated with 65% ammonium sulfate, dissolved in 10 mM Tris-HCl, pH 7.0, and applied to a MonoQ (5/5) column (Amersham Pharmacia Biotech) equilibrated with the same Tris buffer. Fractions eluted with a linear gradient of 0–1.0 M NaCl were concentrated using a Microcon-10 (Millipore, Bedford, MA, USA), and purified by gel filtration on a Superose 6 (10/30) column (Amersham Pharmacia Biotech) equilibrated with PBS. rhSCGF-α

was checked at all stages by Western blotting with the monoclonal antibody KM2142.

N-terminal sequencing of rhSCGF-α

Purified rhSCGF-α was separated on an SDS–PAGE, electrically blotted onto a Problott membrane (PE Applied Biosystems, Foster City, CA, USA), and stained with Coomassie brilliant blue. The rhSCGF-α band was recovered, and tested for automated amino acid Edman sequencing on a model PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan) according to the manufacturer's protocol.

Removal of O-linked oligosaccharides from rhSCGF-α

Purified rhSCGF-α was dissolved in 20 μl of 50 mM sodium phosphate buffer, pH 5.0, and incubated with endo-O-glycosidase (Prozyme, San Leandro, CA, USA) 2 mU and/or sialidase (Boehringer Mannheim, Germany) 20 mU at 37°C for 3 h. The digests were applied to SDS–PAGE and visualized using a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

Other growth factors

Recombinant human cytokines were tested at the following concentrations; GM-CSF (1×10^7 U/mg) and G-CSF (1×10^7 U/mg) at 10 ng/ml unless otherwise indicated, and SCF (5×10^4 U/mg), IL-3 (1×10^7 U/mg), FL and IL-11 at 50 ng/ml [all lyophilized preparations from *E. coli* (PeproTech, Rocky Hill, NJ, USA) were reconstituted with Iscove's modified Dulbecco's medium (IMDM, GIBCO/BRL) containing 10% FCS (Stem Cell Technologies, Vancouver, BC, Canada)].

Bone marrow cells

Human BM cells were aspirated from the sternum of healthy volunteers after informed consent was obtained. BM cells were subjected to a density-cut (<1.077 g/cm^3) by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech) at 400 *g* for 30 min. Low-density cells were collected, and washed twice with IMDM.

Purification of $CD34^+Lin^-$ cells

$CD34^+Lin^-$ cells were separated using a StemSep® kit (Stem Cell Technologies). The low-density BM cells were incubated with a tetrameric antibody complex cocktail, including mouse anti-human CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin A monoclonal antibodies, rat anti-mouse IgG_1 monoclonal antibody and anti-dextran monoclonal antibody, and then with dextran-coated iron colloid beads. Iron particle-bound mature erythroid, myeloid and lymphoid cells were selectively removed in a magnetic field. Purification of $CD34^+Lin^-$ cells, done according to the manufacturer's instructions, yielded $2.2\pm0.3\%$ of starting BM cells with $73.3\pm1.1\%$ purity of $CD34^+$ cells, i.e. about a 50-fold enrichment.

Semisolid clonal culture

Soft agar culture systems for BPA and GPA consisted of 1×10^3 $CD34^+Lin^-$ cells in a 35-mm Nunc Petri dish (triplicate dishes for each group), containing 10 ng/ml rhGM-CSF and 1 U/ml rhEpo (Espo®, Kirin-Sankyo, Tokyo, Japan), respectively, in 1 ml of IMDM supplemented with 20% FCS, 5×10^{-5} M 2-mercaptoethanol, 0.3% Bacto agar and various concentrations of rhSCGF-α with or without a combination of other cytokines. The preparations were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 10 and 14 days, and then scored for GM-colonies and erythroid bursts, respectively.

Short-term liquid culture

Initial liquid culture for primitive hematopoietic progenitor cells consisted of 2×10^4 $CD34^+Lin^-$ cells in 2 ml of IMDM supplemented with 10% FCS unless otherwise indicated, 5×10^{-5} M 2-mercaptoethanol and various concentrations of rhSCGF-α with or without a combination of other cytokines. Whole cells were harvested after a 7-day liquid culture, and were then subjected to a secondary semisolid clonal culture for CFU-GM in the presence of 20 ng/ml rhGM-CSF.

Results

In situ *hybridization*

A longitudinal section of mouse fetus was hybridized with an SCGF antisense probe (Figure 1). Strong signals for expression of SCGF mRNA were distributed around skeletal tissues. These signals were specific for SCGF mRNA, since no signals were detected with a SCGF sense probe (data not shown). To analyse the detailed expression pattern of SCGF mRNA, we further investigated localization of the signals using mouse long bones (Figure 2). Strong signals were observed in BM cells, chondrocytes, the perichondrium, periosteum and primary ossification center-associated cells (Figure 2B). These signals were specific for SCGF mRNA, since no signals were detected with the SCGF sense probe (Figure 2C). In the growth plates, SCGF mRNA was highly expressed in proliferating chondrocytes, but less expressed in resting chondrocytes. In contrast, hypertrophic chondrocytes least expressed SCGF mRNA.

Expression and purification of rhSCGF-α

The concentration of rhSCGF-α secreted into the conditioned medium of pAGE-SCGF-α-transfected CHO cells was estimated at about 3 μg/ml according

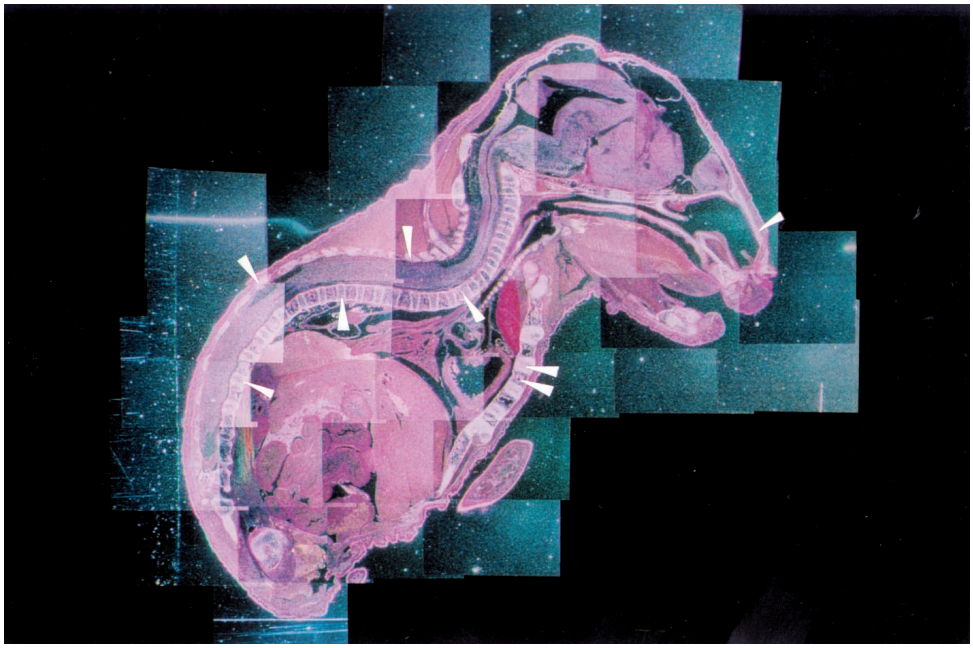

Figure 1 *In situ* hybridization of an 18-day postcoitum mouse fetus with SCGF antisense cRNA. Note white dot signals for expression of SCGF mRNA around skeletal tissues (arrowheads).

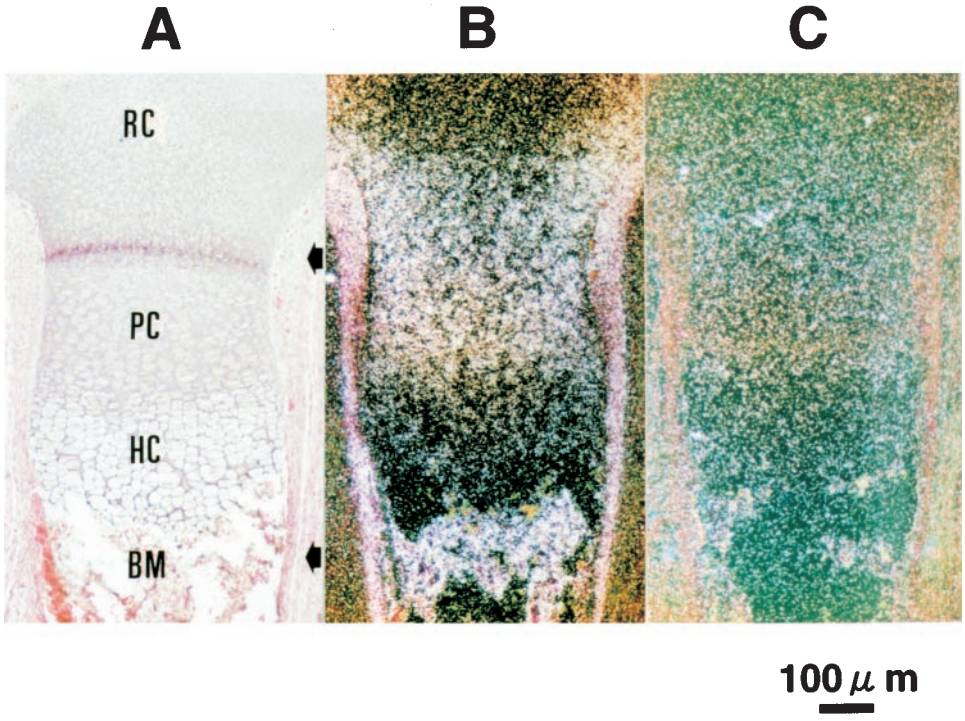

Figure 2 Expression of SCGF mRNA at the proximal end of the tibia of an 18-day postcoitum mouse fetus. Serial sections were subjected to *in situ* hybridization with SCGF [^{35}S]cRNA probes, and counterstained with hematoxylin and eosin. (A) Bright field image. (B) Dark field image with the antisense probe. Expression of SCGF mRNA is conspicuous as white dot signals in bone marrow cells (BM), proliferating chondrocytes (PC), the perichondrium and periosteum (arrows), but less and least notable in resting chondrocytes (RC) and hypertrophic chondrocytes (HC), respectively. (C) Dark field image with the sense probe. The bar represents 100 μm.

to the data from SDS–PAGE and Western blotting. A total of 60 μg of rhSCGF-α was obtained from 190 ml of the conditioned medium through the conventional chromatographies described above. Purified rhSCGF-α was revealed as a single band with a molecular mass of 45 kDa on SDS–PAGE (Figure 3; lane 2). The N-

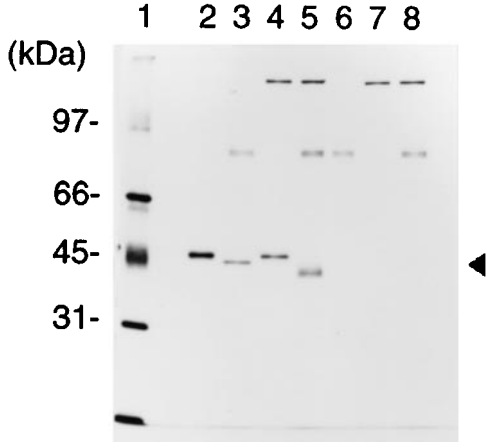

Figure 3 Silver-stained SDS–PAGE of purified rhSCGF-α after digestion with endo-O-glycosidase and/or sialidase. Lane 1, molecular mass markers; lane 2, purified rhSCGF-α; lane 3, rhSCGF-α digested with sialidase; lane 4, rhSCGF-α digested with endo-O-glycosidase; lane 5, rhSCGF-α digested with endo-O-glycosidase in the presence of sialidase; lane 6, sialidase alone; lane 7, endo-O-glycosidase alone; lane 8, endo-O-glycosidase and sialidase. An arrowhead indicates the rhSCGF-α band.

terminal amino acid sequence of purified rhSCGF-α was determined to be ARGAEREWEG, the first alanine of which corresponded with the 22nd alanine of the amino acid sequence deduced from human SCGF-α cDNA.[6] Digestion of rhSCGF-α with endo-O-glycosidase in the presence of sialidase shifted a major band to an apparent molecular mass of 40 kDa (Figure 3; lane 5). Endotoxin contamination in the purified rhSCGF-α preparation was 4.5 ng/mg, equivalent to or below the level of commercially available cytokine preparations (<100 ng/mg).

Hematopoietic activity of rhSCGF-α on $CD34^+Lin^-$ cells

rhSCGF-α exhibited dose-dependent BPA (Figure 4A), GPA (Figure 4B) and CFU-GM-supporting activity during short-term liquid culture (Figure 4C) for $CD34^+Lin^-$ cells, the level of which peaked at over 10 ng/ml rhSCGF-α. rhSCGF-α could not stimulate colony-formation, but achieved low score (10.3 ± 2.5/1×10^3 $CD34^+Lin^-$ cells) small cluster-formation in the presence of Epo. SCGF was cooperative not only with

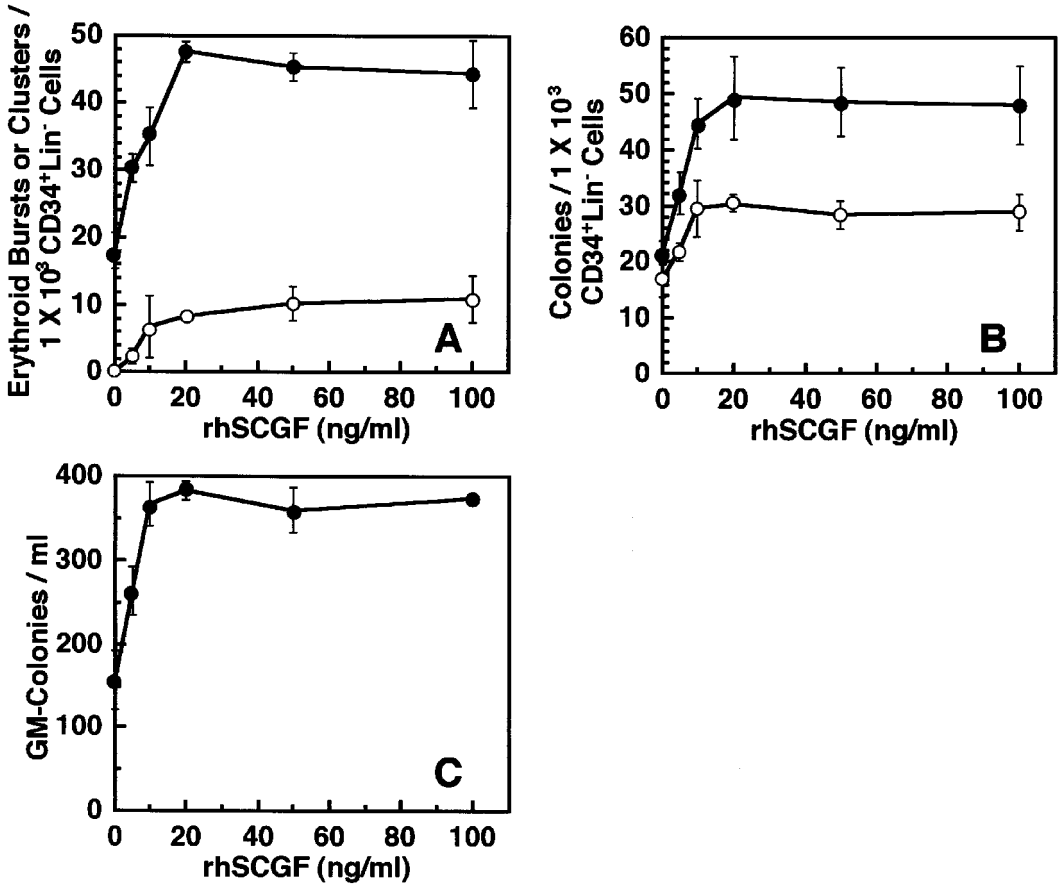

Figure 4 Hematopoietic activity of rhSCGF-α on $CD34^+Lin^-$ cells. Various concentrations of rhSCGF-α were tested for BPA (●) in the presence of 1 U/ml rhEpo (A), GPA in the presence of 10 ng/ml rhGM-CSF (●) or 10 ng/ml rhG-CSF (○) (B) and CFU-GM-supporting activity during short-term liquid culture (C). In (A), a low score of small non-erythroid clusters (○) were formed in addition to erythroid bursts. In (C), the secondary soft agar culture for CFU-GM contained 20 ng/ml rhGM-CSF. Data are shown as means ± s.d. from three separate experiments.

GM-CSF, but also with G-CSF in enhancing colony-growth.

Interaction between SCGF and SCF on BFU-E

It was investigated whether coexistent SCGF and SCF affected BFU-E in the primary soft agar culture of $CD34^+Lin^-$ cells. The BPA of SCF was proportionally suppressed by about 20% with increasing doses of SCGF to the peak level without SCF (Figure 5).

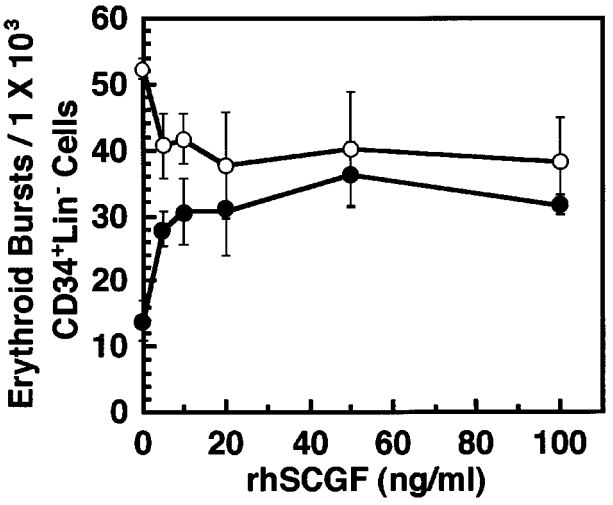

Figure 5 Interaction between SCGF and SCF. Erythroid bursts were scored in the soft agar culture of $CD34^+Lin^-$ cells, containing various concentrations of rhSCGF-α with 1 U/ml rhEpo in the absence (●) and presence (○) of 50 ng/ml rhSCF. Data are shown as means ± s.d. from two separate experiments.

Synergism between SCGF and other cytokines in stimulating hematopoietic colony-formation

Synergistic enhancement of CFU-GM was apparently positive with combinations of SCGF + G-CSF, SCGF + GM-CSF, SCGF + IL-3 and SCGF + FL (Figure 6A). SCGF showed no synergistic enhancement of BFU-E in combination with other cytokines (Figure 6B), but suppressed SCF-stimulated erythroid bursts.

Ex vivo *expansion of CFU-GM during the short-term liquid culture with SCGF and other cytokines*

Further testing to see if SCGF was cooperative with other cytokines to induce *ex vivo* expansion of CFU-GM was done. The FCS concentration in the initial short-term liquid culture was elevated to 20%. A combination of SCGF, IL-3, SCF and FL induced a ninefold *ex vivo* expansion of CFU-GM in $CD34^+Lin^-$ (Figure 7) and a 15-fold increase in non-adherent BM cells (data not shown), as compared with the pre-expansion values.

Discussion

A variety of BM stromal cells produce hematopoietic growth factors and extracellular matrices within complex hematopoietic microenvironments. SCGF is one of such components supporting hematopoiesis, since SCGF mRNA has been reported to be highly expressed in hematopoietic stromal cells.[5,6] The present *in situ* hybridization data indicate that not only BM stromal cells, but also osseous cells themselves could produce SCGF, i.e. expression of

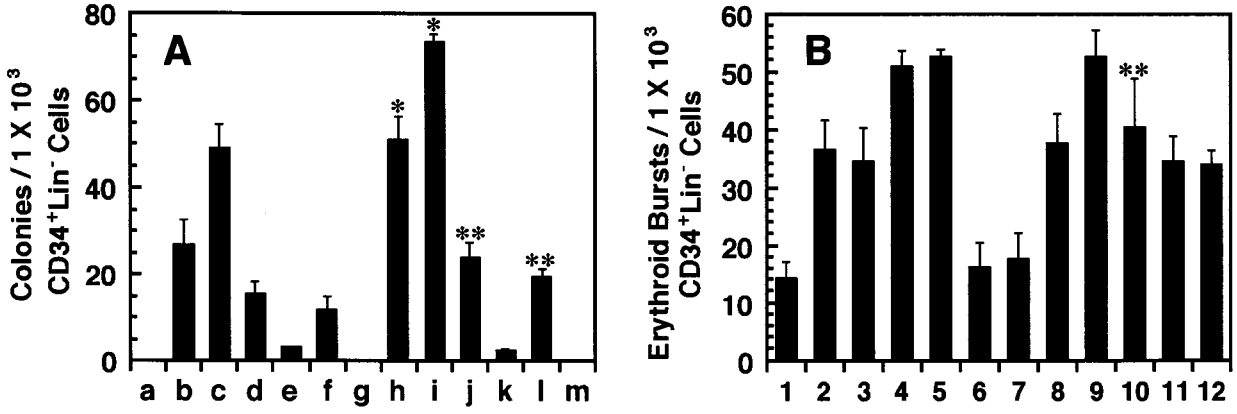

Figure 6 Effect of a combination of cytokines on primary cultures for hematopoietic progenitor cells. (A) $CD34^+Lin^-$ cells were assayed for CFU-GM in a soft agar culture with 50 ng/ml rhSCGF-α (a), 20 ng/ml rhG-CSF (b), 20 ng/ml rhGM-CSF (c), 50 ng/ml rhIL-3 (d), 50 ng/ml rhSCF (e), 50 ng/ml rhFL (f), 50 ng/ml rhIL-11 (g), SCGF + G-CSF (h), SCGF + GM-CSF (i), SCGF + IL-3 (j), SCGF + SCF (k), SCGF + FL (l) or SCGF + IL-11 (m). (B) $CD34^+Lin^-$ cells were assayed for BFU-E in a soft agar culture containing 1 U/ml rhEpo without any cytokines (1), and with 50 ng/ml rhSCGF-α (2), 20 ng/ml rhGM-CSF (3), 50 ng/ml rhIL-3 (4), 50 ng/ml rhSCF (5), 50 ng/ml rhFL (6), 50 ng/ml rhIL-11 (7), SCGF + GM-CSF (8), SCGF + IL-3 (9), SCGF + SCF (10), SCGF + FL (11) or SCGF + IL-11 (12). Each cytokine concentration in the combination of two cytokines is equal with that of a single cytokine. Data are shown as means ± s.d. from two separate experiments. $*P<0.01$, $**P<0.05$, significantly different from the culture with a single cytokine in the combination of two corresponding cytokines.

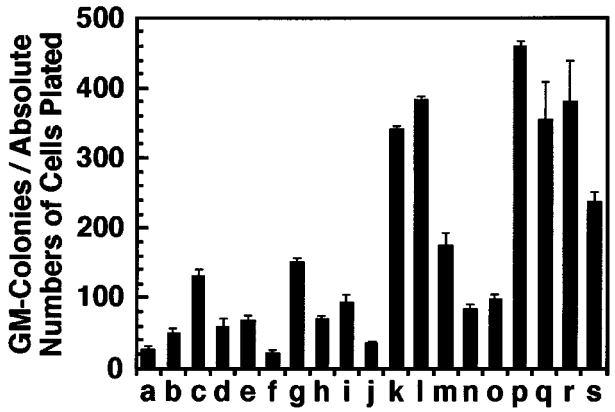

Figure 7 *Ex vivo* expansion of CFU-GM through short-term liquid culture with a combination of cytokines. $CD34^+Lin^-$ cells were cultured in suspension for 7 days in the presence of 20% FCS in IMDM (a), with 50 ng/ml rhSCGF-α (b), 50 ng/ml rhIL-3 (c), 50 ng/ml rhSCF (d), 50 ng/ml rhFL (e), 50 ng/ml rhIL-11 (f), SCGF + IL-3 (g), SCGF + SCF (h), SCGF + FL (i), SCGF + IL-11 (j), SCGF + IL-3 + SCF (k), SCGF + IL-3 + FL (l), SCGF + SCF + FL (m), SCGF + SCF + IL-11 (n), SCGF + FL + IL-11 (o), SCGF + IL-3 + SCF + FL (p), SCGF + IL-3 + SCF + IL-11 (q), SCGF + IL-3 + FL + IL-11 (r) or SCGF + SCF + FL + IL-11 (s). Each cytokine concentration among the combinations of more than two cytokines is equal with that of a single cytokine. The secondary soft agar culture for CFU-GM contained 20 ng/ml rhGM-CSF. The pre-expansion input CFU-GM was $50.3 \pm 6.0/1 \times 10^3$ $CD34^+Lin^-$ cells. Data are shown as means ± s.d. from two separate experiments.

SCGF mRNA is principally confined to skeletal tissues, including BM cells, chondrocytes, primary ossification center-associated cells, the perichondrium and periosteum. The latter two tissues are sites coexpressing collagen I mRNA.[7] Collagen I is necessary for certain cellular interactions in early embryonic hematopoiesis, for site-directed mutagenesis in the mouse collagen I gene induces hematopoietic and mesenchymal cell death.[13] Analysis of SCGF-knockout mice, if available in the future, will be very intriguing to clarify how SCGF acts on hematopoiesis, and also on osteogenesis, as proliferating, but neither resting nor hypertrophic chondrocytes, express SCGF mRNA.

The N-terminal amino acid of purified rhSCGF-α was actually determined to be the 22nd alanine of the amino acid sequence, not the computer-predicted 19th glycine; thus, a 21aa signal peptide MQAAWLLGALVVPQLLGFGHG is cut off to give rise to secreted mature SCGF-α. The calculated molecular mass of mature SCGF-α is 33 534 Da, relatively smaller than that of purified rhSCGF-α at 45 kDa. Because digestion of rhSCGF-α with endo-O-glycosidase and sialidase reduces the molecular mass to 40 kDa, the low mobility of rhSCGF-α produced by CHO cells is due to post-translational modifications such as O-glycosylation, including sialic acids. A proline/serine/threonine-rich PT box at position 91–104[5,6] could be a target site for O-glycosylation.

Hematopoietic activity of rhSCGF-α was confirmed using human BM cells. The resultant data are compatible with those from a previous report,[5] aside from six new findings. First, the SCGF preparation used was not a culture supernatant of transiently SCGF cDNA-expressed COS-1 cells, but purified rhSCGF-α from stably transformed CHO cells with pAGE-SCGF-α. Second, SCGF exhibited BPA, GPA and CFU-GM-supporting activity during short-term liquid culture on $CD34^+Lin^-$ cells. Third, the GPA of SCGF was first demonstrated in the presence of G-CSF. Fourth, SCGF unexpectedly suppressed SCF-stimulated erythroid burst by approximately 20%. Fifth, $CD34^+Lin^-$ cells were stimulated with rhSCGF-α to form rare small clusters. Sixth, synergistic enhancement of CFU-GM was observed in combinations of SCGF and other cytokines, including G-CSF, GM-CSF, IL-3 and FL.

SCGF suppresses SCF-responsive erythroid burst. Why does SCGF suppress the BPA of SCF while itself exhibiting BPA? Some unknown competitive mechanisms must be at work between the SCF-*c-kit* and SCGF-SCGF receptor; the fate of primitive hematopoietic progenitor cells could be modulated by SCGF-induced suppression of SCF-dependent erythroid progenitor cells. TGF-β[14–18] and TNF-α[16–19] reportedly inhibit SCF- and FL-stimulated growth and expansion of primitive hematopoietic progenitor cells, although their action is non-specific.

rhSCGF-α does not exhibit any colony-stimulating activity, but stimulates $CD34^+Lin^-$ cells to form clusters at a plating efficiency of 1%. They have been excluded from colony-counting in the cultures of non-separated BM cells to avoid confusion of overcrowded interspacing single cells with incidental cluster-like cell aggregates. Replating efficiency of the cluster-forming cells and functional studies, including on long-term culture-initiating cells and hematopoietic reconstitution in myeloablative immunodeficient mice, are now in progress to assess the activity of SCGF on hematopoietic stem cell candidates. Flow cytometric studies in our laboratory indicated a significant expansion of another hematopoietic stem cell candidate, $CD34^+CD38^-$ cells, in response to SCGF (manuscript in preparation).

SCGF is synergistic for GPA with G-CSF, GM-CSF, IL-3 or FL, but not with SCF or IL-11. However, no combination of cytokines showed a definite synergism with SCGF for BPA, but BPA in the SCF-containing group was modestly diminished by SCGF. Multiple growth factors reportedly need to cooperate to proliferate and expand hematopoietic progenitor cells 15- to 50-fold; in particular G-CSF, GM-CSF, IL-1, IL-3, IL-6, IL-11 and thrombopoietin are efficient in various combinations with indispensable SCF and FL.[18,20–28] SCGF is not synergistic with other cytokines in inducing *ex vivo* expansion of CFU-GM, although IL-1, IL-6 and thrombopoietin were not tested in the present study.

The potential usefulness of SCGF could be as an early-acting hematopoietic growth factor to build a new hematopoiesis paradigm.

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