Differential Effects of a Stem Cell Factor-Immunoglobulin Fusion Protein on Malignant and Normal Hematopoietic Cells

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ABSTRACT

We genetically connected the extracellular domain of human stem cell factor to the Fc portion of human IgG1. The chimeric recombinant stem cell factor IgG1 fusion protein (rSCF-IgG1) had an apparent $M_r$ of 190,000 and consisted of three identical covalently linked subunits. It specifically bound to c-kit and the high affinity Fc receptor, respectively. Liquid phase rSCF-IgG1 was found to have a higher affinity for ligand binding than native human rSCF. The effect of liquid phase rSCF-IgG1 on liquid phase $[^3H]$thymidine uptake was comparable to the plateau level of [methyl-$^3H$]thymidine uptake by malignant cells was decreased by the latter, whereas proliferation of nonmalignant progenitor cells was supported. Liquid phase rSCF-IgG1 caused enhanced and prolonged receptor phosphorylation and a more rapid down modulation of c-kit. Our data support the concept that solid phase attachment of rSCF-IgG1 is sufficient for alteration of biological function and that rSCF-IgG1 partially blocks SCF-stimulated malignant cell growth while supporting normal progenitor cells.

INTRODUCTION

SCF,$^3$ which is predominantly produced by bone marrow stromal cells, and its receptor c-kit play a central role in proliferation and differentiation of hematopoietic progenitor cells (1). In addition, SCF is an essential cytokine for short-term ex vivo expansion of CD34-positive PBSCs (2) and has been shown to have CD34-positive cells mobilizing potential when administered to patients scheduled for autologous stem cell transplantation (3). In serum, SCF predominantly circulates as a biologically inactive monomeric molecule. Only 10% represent noncovalently linked dimers mediating the biological function (4). Alternative splicing and specific proteolytic cleavage lead to a soluble and a membrane-bound SCF isoform. Mice with the $^{Sl}d$ mutation causing a deletion of the transmembrane and cytoplasmic domains of SCF are sterile, have severe macrocytic anemia, and reduced tissue mast cells (5, 6). These findings indicate that soluble SCF cannot compensate for membrane-bound SCF and suggest essential distinct functions of both isoforms. It is not clear, however, if membrane-attachment of SCF alone, other cytokines, and adhesion molecules acting in concert with membrane-bound SCF, or absent reverse signaling mediated by the cytoplasmic domain of transmembrane SCF account for the $^{Sl}d$ phenotype.

It is well established that bone marrow stroma cells have differential effects on malignant versus nonmalignant hematopoietic progenitors. In chronic myeloid leukemia, for example, Philadelphia chromosome-positive tumor cells typically decline during the first few weeks of LTC, whereas nonmalignant progenitor cells may then become detectable (7). Very primitive normal progenitor cells, identified by their ability to give rise to colonies after 5 weeks or more in culture, are supported by a narrow feeder layer and are, thus, referred to as LTC-initiating cells (8). It is unclear as to what extent stroma cell-expressed SCF contributes to this differential effect. Addition of a c-kit-specific antibody does not cause a complete loss of primitive progenitors, suggesting that other cytokines produced by the feeder cells also support stem cell survival (9). In these stroma cell-based culture systems, the role of membrane-bound SCF cannot stringently be assessed. With this background, there were three major intentions to construct the rSCF-IgG1 fusion protein, as done in this study: (a) the Fc portion facilitates easy binding to solid phase via Fc-specific antibodies. The influence of solid phase attachment on the biological function of human SCF can, thus, be studied in a clean experimental system; (b) we wanted to know whether rSCF-IgG1 had differential effects on malignant versus nonmalignant hematopoietic cells similar to stroma cell function; (c) linking the SCF protein to the Fc portion of IgG1 offers the potential advantages of introducing intermolecular disulfide bonds to increase biological activity and, potentially, to prolong in vivo half-life due to decreased renal elimination, as a result of increased molecular weight.

MATERIALS AND METHODS

Cell Lines. The human myelomonocytic leukemia cell line U937, the murine myeloma cell line J558, and CS-1, a c-kit-positive cell line derived from blasts of an acute myeloid leukemia patient, were propagated in standard culture medium consisting of RPMI-1640 with 10% FCS (both from Life Technologies, Inc., Gaithersburg, MD), 1 mM sodium pyruvate, and 2 mM l-glutamine (both from Biochrom, Berlin, Germany). For the human acute megakaryoblastic leukemia cell line M-07e, 1 unit/ml granulocyte-colony stimulating factor (Sandoz, Nuernberg, Germany) and 20% FCS were added. The human adenocarcinoma cell line SK-Hep1 and the green monkey kidney cell line COS-1 were maintained in DMEM (Life Technologies, Inc.) completed with 10% FCS, 1 mM sodium pyruvate, and 2 mM l-glutamine. Adherently growing cells were detached with treatment by 0.05% trypsin/0.02% EDTA solution (Biochrom). Routinely, cells were refed by medium exchange every 3 or 4 days and cultured in a humidified atmosphere at 37°C and 5.5% CO$_2$. Bisbenzimide staining for mycoplasma contamination (Biochrom) was performed every 2–4 weeks. Positive cells were discarded.

Cloning, Expression, and Purification of rSCF-IgG1. A DNA for human SCF was reverse transcribed from RNAzol-B (WAK-Chemie, Bad Homburg, Germany) prepared total RNA of primary blasts of a chronic myeloid leukemia patient and of SK-Hep1 cells. The Perkin-Elmer standard protocol for PCR (Roche Molecular Systems, Branchburg, NJ) was used for amplification of the cDNA. Sense-(5′-GGCGCTGCTCCGAGTATGAGAAAGACA-CAAACCTTG-3′) and antisense-(5′-GCTGGCAATGCCATGGGAGGTCCCATGTTAGCCTGGAGT-3′) primers (TIB Molbiol, Berlin, Germany) were chosen to flank the sequences coding for the 25 amino acids of the natural signal peptide, for 189 amino acids of the extracellular domain of the soluble human SCF (10), and to introduce appropriate restriction sites for directed

Received 10/23/98; accepted 4/19/99.

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1 Supported by the Deutsche Krebshilfe (W10/94 N02), the Deutsche Forschungsgemeinschaft (SFB 506 TP C4), and a grant from the Sonnensfeld-Stiftung.

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3 The abbreviations used are: SCF, stem cell factor; rSCF, recombinant SCF; rSCF-IgG1, chimeric rSCF-IgG1 protein; IL, interleukin; IL-2-IgG1, chimeric recombinant IL-2-IgG1 fusion protein; mAb, monoclonal antibody; PBSC, peripheral blood-derived hematopoietic progenitor cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase.

4 The abbreviations used are: SCF, stem cell factor; rSCF, recombinant SCF; rSCF-IgG1, chimeric rSCF-IgG1 protein; IL, interleukin; IL-2-IgG1, chimeric recombinant IL-2-IgG1 fusion protein; CFU, colony-forming unit; EC$_{50}$, effective dose conferring half maximum of [methyl-$^3H$]thymidine uptake; FcγRI, high affinity Fcγ receptor; LTC, long-term culture; mAb, monoclonal antibody; PBSC, peripheral blood-derived hematopoietic progenitor cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate; a.u., arbitrary unit.

5 The abbreviations used are: SCF, stem cell factor; rSCF, recombinant SCF; rSCF-IgG1, chimeric rSCF-IgG1 protein; IL, interleukin; IL-2-IgG1, chimeric recombinant IL-2-IgG1 fusion protein; mAb, monoclonal antibody; PBSC, peripheral blood-derived hematopoietic progenitor cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate; a.u., arbitrary unit.

6 The abbreviations used are: SCF, stem cell factor; rSCF, recombinant SCF; rSCF-IgG1, chimeric rSCF-IgG1 protein; IL, interleukin; IL-2-IgG1, chimeric recombinant IL-2-IgG1 fusion protein; mAb, monoclonal antibody; PBSC, peripheral blood-derived hematopoietic progenitor cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate; a.u., arbitrary unit.
coding. The cDNA product was cloned by standard procedures into a pCDM8-derived expression vector, which already contained the hinge-, CH2-, and CH3-domains of human IgG1 with the intervening introns and was kindly provided by Brian Seed (11). The rSCF-IgG1 insert was recloned into pcDNA3 (Invitrogen, Leek, the Netherlands) and transfected into J558 cells by electroporation (Bio-Rad Laboratories, Richmond, CA). A selected G418-resistant clone, producing 1 µg of rSCF-IgG1/day and 10^8 viable cells as measured by a previously described enzyme-linked immunosorbent assay for human IgG1 (12), was continuously cultured in a hollow fiber cartridge (Cellco, German- town, MD). rSCF-IgG1 was purified from culture supernatants of COS-1 or J558 cells by ammonium sulfate precipitation, desalting by gel filtration using Sephadex G-25, and affinity chromatography using Protein-A-Sepharose (both from Pharmacia, Uppsala, Sweden), according to standard protocols.

**Size Determination and Molar Concentration of the Purified rSCF-IgG1 Preparations.** Samples of 5 µg of purified rSCF-IgG1 were reduced with 2-mercaptoethanol or were left untreated. Proteins were electrophoretically separated on a 5–20% SDS-PAGE according to Laemmli (13). Western blot analysis was carried out with a SCF-specific antibody (R&D Systems, Minneapolis, MN) or with an IgG1(Fc)-specific antibody (Sigma Chemical Co., St. Louis, MO). Binding of the peroxidase-conjugated secondary antibody (Immunoresearch, West Grove, PA) was nonradioactively detected (Amer- sham, Little Chalfont, United Kingdom). Yeast-derived rSCF (Genzyme, Cam- bridge, MA), and human immunoglobulin (Biostech, Dreieich, Germany) served as positive controls. M_r of nonreduced and reduced rSCF-IgG1, respectively, was calculated in relation to prestained standard molecular weight marker proteins (Sigma Chemical Co.). Molar concentrations of rSCF-IgG1 final preparations were calculated from the Mc determined for the nonreduced protein and the concentration determined by the enzyme-linked immunosorbent assay for human IgG1.

**Competitive Binding Assay.** U937 and M-07e cells were incubated with 10 nM rSCF-IgG1 or remained untreated. For competitive displacement studies, U937 cells were incubated with the fusion protein alone or added to a 10-fold molar excess of a neutralizing mAb specific for the FcγRI (clone 197; Medarex, Annandale, NJ). M-07e cells were incubated with 3.3 µg biotinyl- rated rSCF (R&D Systems) alone or with a 10-fold molar excess of rSCF- IgG1. Surface-bound fusion protein and biotinylated rSCF were detected by flow cytometry (Becton Dickenson, San Jose, CA) using a FITC-conjugated secondary antibody (Immunotech, Marseille, France) and FITC-conjugated avidin (R&D Systems), respectively. Receptor expression on U937 and M-07e cells was visualized using a FcγRI-specific mAb (clone 32.2; Medarex) and the c-kit-specific mAb (clone 95C3) visualized by a FITC-conjugated secondary antibody (all from Immunotech).

**Cell Proliferation Assays.** M-07e cells were starved as described previ- ously (14). Flat bottom tissue culture plates (96-well) were precoated with 10 µg/ml IgG1(Fc)-specific antibody in phosphate-buff- ered NaCl solution for 1 h at 37°C. Nonspecific binding was blocked overnight at 4°C with 0.1% BSA in the same buffer. Triplicates of equimolar concentra- tions of rSCF-IgG1, rIL-2-IgG1, and yeast-derived rSCF, respectively, were titrated to untreated and pretreated wells. Tissue culture plates were incubated for 1 h at 37°C, and 2 × 10^5 cells were added to each well. Several wells were washed extensively before adding the cells. Microcultures received 1 µCi [methyl-^3]Hthymidine (Amersham, Little Chalfont, United Kingdom) per well for the last 4 of 72 h of culture and were frozen at −80°C for at least 4 h afterwards. Incorporated [methyl-^3]Hthymidine was harvested to a glass fiber membrane after rapid thawing of the cultures at 37°C and detected by liquid scintillation counting (LKB Wallac, Turku, Finland). Analogous experiments were performed with c-kit positive CS-1 cells and CD34-positive PBSCs. Kinetics of CS-1 proliferation were estimated by adding 1 µCi of [methyl-^3]Hthymidine/well to the microcultures during the last 4 hours before harvesting after various time points, as described previously.

**Cell Adhesion Assay.** Tissue culture plates (96-well) precoated with the IgG1(Fc)-specific antibody, as described previously, were incubated with 10 nM rSCF-IgG1 for 1 hour at 37°C. After extensive washing, 2 × 10^5 CS-1 or factor-starved M-07e cells were added to each well. At various time points, microcultures were checked for cell adhesion, which was monitored by phase contrast microscopy (Leitz, Wetzlar, Germany). Standard growth medium and 10 nM rSCF served as controls.

**Purification of Nonmalignant CD34-positive Progenitor Cells.** PBSCs were prepared from peripheral blood of a breast cancer patient after mobilization by chemotherapy and granulocyte-colony stimulating factor treatment. Informed consent was obtained from all patients in the study. The peripheral blood mononuclear cell fraction was obtained from heparinized whole blood by Hypaque-Ficoll (Pharmacia) density gradient centrifugation (P = 1.078 g/ml). CD34-positive PBSC fraction was isolated according to the Miltenyi standard protocol for positive magnetic cell selection using a CD34-specific mAb (clone QBEND10; Miltenyi Biotec, Sunnyvale, CA). Purity of the CD34-positive progenitor cell preparation was checked by flow cytometry with PE-conjugated CD34-specific mAb (clone 8G12, Becton Dickinson). Viability testing of thawed PBSCs was done using propidium iodide (ICN, Aurora, OH) staining.

**17C of Nonmalignant CD34-positive Progenitor Cells.** Freshly prepared or thawed PBSCs (1 × 10^6) with an initial purity of about 90% CD34-positive cells were seeded with 50 µM liquid phase rSCF or rSCF-IgG1 in standard growth medium to a 24-well tissue culture plate (Nunc, Roskilde, Denmark). Standard growth medium served as negative control. Every 7 days, cultures were refed with the appropriate fresh medium. After 43 days, remaining cells were microscopically counted and seeded into an assay detecting CFUs.

**CFU Assay.** 1 to 5 × 10^3 freshly prepared or thawed CD34-positive PBSCs (1 to 5 × 10^3) and 10^3 to 10^4 cells from LTC, respectively, were seeded with 1 ml of solidified MethoCult medium (Stem Cell Technologies, Vancouve- r, Canada) to tissue culture dishes (2.5 mm; Nunc). After 14 days, cultures were microscopically examined for CFU formation.

**Phosphorylation of c-kit.** Factor-starved M-07e cells were incubated for 30 min with increasing concentrations of rSCF or rSCF-IgG1 and for various periods of time with 10 nM rSCF and rSCF-IgG1, respectively. In each case, 10^5 cells were harvested, washed once with phosphate-buffered NaCl solution, and lysed according to Nelson et al. (15) with a lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 1% TritonX-100, 150 mM sodium chloride, and 1 mM phenylmethyl-sulfonyl fluoride (all from Merck) and 1 mM sodium orthovanadate and 5 mM EDTA (both from Sigma Chemical Co.). Protein content of the total cell lysates was determined using Bradford reagent (Bio- Rad Laboratories). Total cell lysates corresponding to 5 × 10^5 M-07e cells were incubated overnight under constant agitation with 10 µg of c-kit-specific mAb (clone YB5.8; PharMingen, Los Angeles, CA) preloaded to Protein-G-Sepharose beads (Sigma Chemical Co.). Beads were washed twice with 1% TritonX-100 in 100 mM Tris-HCl (pH 8.0) and resuspended in reducing loading buffer for SDS-PAGE. All steps were carried out at 4°C, and all solutions used were prechilled on ice. Phosphorylation of c-kit was detected by Western blot, as described previously, with a phosphotyrosine-specific mAb (clone PT-66; Sigma Chemical Co.) and peroxidase-conjugated secondary antibody (Immunoresearch).

**Modulation of c-kit.** Factor-starved M-07e cells were incubated for 1 hour at 37°C in 6-well tissue culture plates (Nunc) without any cytokine or with 1 nM rSCF and rSCF-IgG1, respectively. The rSCF-IgG1 remained in liquid phase or was precoated with the IgG1(Fc)-specific antibody, as described previously. After incubation, the adherent cell fraction was released using a cell scraper, and all cells were collected for estimating c-kit expression by flow cytometry using a PE-labeled-c-kit-specific mAb (clone 95C3; Immunotech).

**RESULTS**

The Purified rSCF-IgG1 Expressed by Stably Transfected Murine Myeloma Cells Is a 190-kDa Fusion Protein That Consists of Three Identical Covalently Linked Subunits Specifically Binding to FcγRI and c-kit. In Western blot analysis, the rSCF-IgG1 was detected by antibodies specific for human SCF and the Fc portion of human IgG1 (Fig. 1), respectively. Under nonreducing conditions the fusion protein formed a single band corresponding to 190 kDa. Reduction of the rSCF-IgG1 resulted in a single band of about 65 kDa. Partial reduction of the fusion protein produced an additional band of 130 kDa (data not shown). The rSCF was not affected by 2-mercap- toethanol. Native human immunoglobulin was totally reduced from a pattern of distinct protein bands to a single band representing the immunoglobulin heavy chain. These data suggested a complex rSCF- IgG1 molecule formed by three identical subunits, which are covalently linked by intermolecular disulfide bonds. The rSCF-IgG1

2925
SCF specific antibody  IgG1(Fc) specific antibody

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Fig. 1. rSCF-IgG1 is a trimeric molecule with an apparent M, 190,000. The rSCF-IgG1 (Lanes 2, 3, 6, and 7) was separated on a 5–20% gradient SDS-PAGE and detected by Western blot analysis using antibodies specific for human SCF (lanes 1–4) or IgG1(Fc) (lanes 5–8). rSCF (lanes 1 and 4) and human immunoglobulin (Lanes 5 and 8) served as positive controls. All proteins were loaded under nonreducing (Lanes 1, 2, 3, and 6) and reducing conditions (Lanes 3, 4, 7, and 8). M, markers are indicated.

preparation mainly consisted of the trimeric form of the molecule. Therefore, molar concentrations were calculated based on a M, 190,000.

Cells that express the receptors for either the IgG1 or the SCF portion of the rSCF-IgG1 were used to test the binding properties of the fusion protein. Binding of the rSCF-IgG1 to FcγRI-positive U937 cells and to c-kit-positive M-07e cells resulted in a pronounced shift of FI compared with the negative control (Fig. 2, A and B). The fluorescence profile obtained was superimposable to that of the FcγRI and the c-kit expression, respectively. Binding of rSCF-IgG1 to FcγRI was blocked by a 10-fold molar excess of a neutralizing FcγRI specific mAb (Fig. 2C). Coincubation of M-07e cells with biotinylated rSCF and a 10-fold molar excess of rSCF-IgG1 inhibited binding of the biotinylated rSCF (Fig. 2D). These in vitro findings indicated a bifunctional rSCF-IgG1 fusion protein.

Solid Phase-bound rSCF-IgG1 Has Differential Effects on c-kit-positive Malignant versus Nonmalignant Cells. Factored-starved M-07e cells were cultured with increasing concentrations of rSCF, rSCF-IgG1, and rIL-2-IgG1, respectively (Fig. 3). The half maximum level of [methyl-3H]thymidine uptake was induced by 95 pm rSCF-IgG1 compared with 820 pm rSCF. Three independent experiments suggested that liquid phase rSCF-IgG1 on a molar basis was about 8 times more potent than rSCF in stimulating proliferation of this c-kit-positive malignant cell line. Although the EC50 values of liquid phase and matrix coupled rSCF-IgG1 were comparable, the plateau proliferation level was reduced by 30–40% in the presence of the solid phase-bound fusion protein (Fig. 3, B and C). Similar experiments were performed with the c-kit positive factor-independent CS-1 cell line derived from blasts of an acute myeloid leukemia patient and with nonmalignant CD34-positive PBSCs obtained from a breast cancer patient. The maximum levels of [methyl-3H]thymidine uptake and the EC50 values conferred by rSCF and rSCF-IgG1, respectively, are summarized in Table 1. The rSCF-IgG1 had approximately 8-fold lower EC50 values for all cell samples (M-07e, 8.63; CS-1, 8.44; PBSC, 8.00). Coupling of the rSCF-IgG1 to a solid phase did not affect the EC50 values. Matrix-bound rSCF-IgG1 reduced the maximum growth potential of malignant cells. Proliferation of growth factor-dependent M-07e cells was decreased to 62–68% of control with liquid phase rSCF-IgG1. Growth of factor-independent CS-1 cells was slightly, but significantly, reduced to 88–91% of control. In contrast, nonmalignant PBSCs proliferated to 109–115% of control under identical experimental conditions. These data indicate that solid phase-bound rSCF-IgG1 has preferential growth-retarding effects on malignant cell lines while promoting growth of nonmalignant hematopoietic cells.

Solid Phase-bound rSCF-IgG1 Partially Blocks SCF-stimulated Malignant Cell Growth. We next addressed if partial blockage of SCF-stimulated malignant growth by solid phase-bound rSCF-IgG1 was time-dependent. CS-1 cells were cultured for various periods of time with 500 pm rSCF-IgG1 that has been found to produce maximum growth (Fig. 4). Cells slightly proliferated without any cytokine during this time period and were not affected by the matrix-coupled IgG1(Fc) specific antibody. The addition of rSCF-IgG1 stimulated
proliferation to a 2.5-fold higher maximum level of [methyl-3H]thymidine uptake after 24 hours. In the presence of liquid phase rSCF, maximum proliferation continued at a constant rate during the whole culture period, whereas the solid phase-bound rSCF-IgG1 partially blocked SCF-dependent proliferation after 24–72 hours. These experiments indicate that growth inhibition takes about 72 hours and may be effective for at least 80 hours.

**Solid Phase-bound rSCF-IgG1 Causes an Adherence-dependent Shape Change of c-kit-positive CS-1 Cells.** The rSCF-IgG1 coated to tissue culture dishes mediated adherence of c-kit-positive M-07e and CS-1 cells. Although M-07e cells remained nearly spherical, 60–80% of the CS-1 cells became spindle-shaped (Fig. 5, B and D). Adherence was transient and lasted about 24 hours. Cells that were cultured in standard medium alone (Fig. 5, A and C) or with rSCF (data not shown) remained spherical and in suspension.

**Liquid Phase rSCF-IgG1 Has an Increased Potential to Maintain Primitive Nonmalignant Hematopoietic Progenitor Cells.** Nonmalignant CD34-positive PBSCs with an initial purity of 90% were maintained in stroma-free LTC (Table 2). In each case, the total CFU number recovered after 43 days of culture dramatically decreased from initially $8.2 \times 10^5 \pm 1155$. In the presence of 50 pm rSCF, $869 \pm 132$ CFUs remained versus $2176 \pm 248$ CFUs with equimolar concentrations of the rSCF-IgG1. This significantly higher CFU recovery in the presence of rSCF-IgG1 indicates an increased potential to maintain primitive progenitor cells.

**Liquid Phase rSCF-IgG1 Enhances and Prolongs c-kit Phosphorylation.** We hypothesized that the higher proliferative activity of rSCF-IgG1 compared with rSCF was related to changes in the receptor phosphorylation kinetics. In the presence of 5 nm rSCF, the degree of c-kit phosphorylation was comparable with that obtained by a 100-fold lower molar concentration of rSCF-IgG1 (Fig. 6A). For comparison of phosphorylation kinetics 10 nm rSCF and rSCF-IgG1, respectively, were used. In the presence of rSCF, maximum c-kit phosphorylation was observed after 30 min and disappeared after 240 min (Fig. 6B). In contrast, maximum phosphorylation caused by liquid phase rSCF-IgG1 was already obtained after 5 min, followed by a more delayed decrease during the following 480 min. In addition, the maximum intensity of phosphorylated bands was 2.5-fold more pronounced.

**The rSCF-IgG1 Is More Effective in Down Modulation of c-kit Compared with Native rSCF.** M-07e cells that were incubated for 1 hour without any cytokine expressed c-kit at a high level. rSCF decreased c-kit expression to 244.1 a.U. (Fig. 7B) compared with 353.2 a.U. of the control (Fig. 7A). In the presence of liquid phase-(Fig. 7C) or solid phase-bound rSCF-IgG1 (Fig. 7D), receptor density was decreased to a FI of 70.1 a.U. and 61.7 a.U., respectively. These data indicate a rapid and more pronounced down modulation of c-kit by the fusion protein.

**DISCUSSION**

The hematopoietic support provided by a fibroblast cell line transfected with the membrane-bound human SCF was found to be superior to soluble SCF in vitro (16). Impaired hematopoiesis is observed in vivo in SIR/SP mice lacking membrane-bound SCF (17). Both findings represent circumstantial evidence for different biological functions of the SCF isoforms. In these experiments, however, confounding factors such as other stroma cell-produced cytokines, local adhesion molecules of the bone marrow matrix, or other cellular elements cannot be excluded. We, therefore, constructed rSCF-IgG1, which can easily be attached to a solid-phase in a directed fashion. Solid-phase attachment of rSCF-IgG resulted in a decrease of the proliferation plateau level of SCF-dependent M-07e cells by 50% and in partial blockage of c-kit-stimulated growth of SCF-independent CS-1 cells. These inhibitory effects on SCF-stimulated proliferation were not due to limiting concentrations of rSCF-IgG1, but could be explained by a more pronounced modulation of c-kit from the cell surface. In addition, rSCF-IgG1, but not rSCF, induced firm plastic adherence of malignant M-07e and CS-1 cells. It has been shown that rSCF provides an inside-out signal that increases the affinity of VLA-4 and VLA-5 integrins for fibronectin (18) and of the vascular cell adhesion molecule-1 (19). In contrast to the growth-promoting effect after SCF-mediated enhanced integrin avidity, we observed a reduction of proliferation. It is known that receptor crosstalk occurs between c-kit and the focal adhesion kinase pp125FAK (20). Other growth-affecting
signaling pathways may specifically be influenced by rSCF-IgG1 and need to be investigated in our system. In summary, the refined experimental systems obtained using rSCF-IgG1 provide clear-cut evidence that altered spatial organization of SCF in itself is sufficient to cause different biological functions.

Although the natural leader sequence of SCF has been used, satisfactory production rates of rSCF-IgG1 were achieved in J558 cells, a professional antibody-producing murine myeloma cell line (21), but not in COS-1 cells (data not shown). Similar observations were made for the production of murine IL-15-IgG2b (22). We included the whole 189 amino acid extracellular domain containing the proteolytic cleavage site encoded by exon 6 to allow for adequate spacing and proper folding of the chimeric molecule. Native soluble SCF has two intramolecular disulfide bonds between cysteine residues 4-89 and 43-138 and circulates partially as a noncovalently bonded dimer (23). The high dissociation rate and loss of activity had been attempted to overcome by covalently linking SCF monomers. Along these lines, Lu et al. (24) have produced the NH2-terminal 165 amino acid sequence in Escherichia coli inclusion bodies. Oxidation and in vitro refolding yielded SCF dimers in which the intrinsic cysteine residues were involved in intermolecular disulfide bonding. The purified material showed 3-fold higher biological activity in vitro. Before extensive purification, however, there were still 90% inactive dimers and monomers present. We assumed our strategy of hinge-dependent dimerization to have the theoretical advantage of not influencing the three-dimensional cytokine structure. Unexpectedly, rSCF-IgG1 was produced as a trimeric molecule consisting of identical 65 kDa subunits, an rSCF isoform that has not been reported before. These findings suggest that our construct leads to intermolecular polymerization possibly involving cysteine residues of the hinge-region in the Fc-portion. Correspondingly, on a molar basis, rSCF-IgG1 was about eight times more potent in stimulating growth factor-dependent

Table 2

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<td>50 µl rSCF-IgG1</td>
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* P < 0.001 compared by ANOVA and subsequent Bonferroni-adjusted multiple t tests compared with rSCF.
M-07e cells compared with rSCF. Altered cell surface receptor interactions seemed not to be responsible for that because receptor avidity was not significantly increased (data not shown). However, we found profoundly enhanced and prolonged c-kit phosphorylation induced by rSCF-IgG1. More persistent tyrosine kinase activation has also been described for stimulation with transfected membrane-bound SCF compared with its soluble form (25). In addition, c-kit protein, which normally becomes degraded by polyubiquitination, had a longer half-life when stimulated with membrane-bound SCF (26). These mechanisms may also be involved in the increased biological activity of rSCF-IgG1.

In vitro culture on a stroma cell layer is used as a purging technique in autologous bone marrow transplantation for myeloid leukemias (27, 28). Due to the ease of leukapheresis, peripheral blood is increasingly being used as a stem cell source, which has the disadvantage of lacking stroma cell elements that could be used for purging. In view of using rSCF-IgG1 as a potential component of an artificial bone marrow matrix in this setting, we studied its effect on both malignant and nonmalignant hematopoietic cells. SCF-dependent proliferation of M-07e cells and of the monoblastic cell line CS-1 was significantly reduced by solid phase-bound rSCF-IgG1. This observation is consistent with the capacity of bone marrow stroma cells to block leukemic differentiation and promote apoptosis of leukemia cells in vitro (29). Conceptually, leukemic cells may have a selection advantage as a result of reduced adherence, thereby escaping stroma cell-dependent growth inhibition. Our data on CS-1 cells indicate that rSCF-IgG1 may partially correct this adhesion deficiency of c-kit-positive tumor cells and, thus, may contribute to their elimination in vitro.

Interfering with an autocrine loop seems unlikely because CS-1 cells fail to produce SCF (data not shown). In contrast, rSCF-IgG1 supported proliferation of nonmalignant CD34-positive PBSCs in both short-term and long-term assays. SCF is known to play a pivotal role for the self-renewal of early human hematopoietic progenitor cells (16). The rSCF-IgG1 seemed superior to rSCF in supporting LTC-initiating cells. Taken together, the rSCF-IgG1 has differential effects on malignant cell lines versus nonmalignant hematopoietic progenitor cell growth resembling the properties of a stroma cell layer. It may, thus, become a substitute for membrane-bound SCF and a central

![Fig. 6. Dose-response relationship and kinetics of c-kit phosphorylation induced by rSCF and rSCF-IgG1, respectively. Anti-c-kit immunoprecipitates of M-07e cell lysates were separated under reducing conditions in a 5–20% gradient SDS-PAGE. Western blot analysis was performed using an antiphosphotyrosine mAb. Equal protein amounts estimated before immunoprecipitation were loaded. The position of the c-kit protein at 145 kDa is indicated by arrows. A, M-07e cells were treated for 30 min with increasing concentrations of the appropriate cytokine. B, cells were harvested before and at various time points after the addition of 10 ng rSCF and rSCF-IgG1, respectively.](image)

![Fig. 7. Modulation of c-kit by rSCF-IgG1 compared with native rSCF. M-07e cells were incubated for 1 h at 37°C without any cytokine (A), with 1 nM rSCF (B), with 1 nM liquid phase rSCF-IgG1 (C), and 1 nM solid phase-bound rSCF-IgG1 (D), respectively. c-kit expression was detected by flow cytometry using a PE-labeled c-kit-specific mAb (clone 95C3). Negative control is shown in A as a dotted line. For the marked region, FI in a.U. is indicated individually.](image)
component of a completely defined synthetic matrix being used to engineer tumor-free autografts.

IgG-Fc fusion proteins represent a new class of therapeutics that have entered clinical testing. A human tumor necrosis factor receptor (p75)-Fc fusion protein was found to be a safe and effective agent in controlling rheumatoid arthritis activity, with a rapid onset of action at surprisingly low doses of 16 mg/m² body surface area administered twice weekly (30). The rSCF-IgG1 is expected to have low immunogenicity and a prolonged serum half-life due to its size preventing renal elimination, as was shown for CTLA4-IgG used for immunosuppression in vivo (31). We have constructed rSCF-IgG1 with unique biochemical properties having not been described before. This molecule allows to clearly relate differential biological functions to alterations in molecular structure or spatial orientation. It may gain relevance in autologous stem cell transplantation for both in vitro and in vivo applications.

ACKNOWLEDGMENTS

We thank T. Blankenstein, in particular, for critical reading of the manuscript, and we are grateful to U. Kunzendorf for helpful discussions. We thank J. Maurer for sequencing the DNA construct.

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Differential Effects of a Stem Cell Factor-Immunoglobulin Fusion Protein on Malignant and Normal Hematopoietic Cells

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