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Design of recombinant stem cell factor–macrophage colony stimulating factor fusion proteins and their biological activity *in vitro*

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Summary

Stem cell factor (SCF) and macrophage colony stimulating factor (M-CSF) can act in synergistic way to promote the growth of mononuclear phagocytes. SCF–M-CSF fusion proteins were designed on the computer using the Homology and Biopolymer modules of the software packages InsightII. Several existing crystal structures were used as templates to generate models of the complexes of receptor with fusion protein. The structure rationality of the fusion protein incorporated a series of flexible linker peptide was analyzed on InsightII system. Then, a suitable peptide GGGGSGGGGSGG was chosen for the fusion protein. Two recombinant SCF–M-CSF fusion proteins were generated by construction of a plasmid in which the coding regions of human SCF (1–165aa) and M-CSF (1–149aa) cDNA were connected by this linker peptide coding sequence followed by subsequent expression in insect cell. The results of Western blot and activity analysis showed that these two recombinant fusion proteins existed as a dimer with a molecular weight of ~84 KD under non-reducing conditions and a monomer of ~42 KD at reducing condition. The results of cell proliferation assays showed that each fusion protein induced a dose-dependent proliferative response. At equimolar concentration, SCF/M-CSF was about 20 times more potent than the standard monomeric SCF in stimulating TF-1 cell line growth, while M-CSF/SCF was 10 times of monomeric SCF. No activity difference of M-CSF/SCF or SCF/M-CSF to M-CSF (at same molar) was found in stimulating the HL-60 cell linear growth. The synergistic effect of SCF and M-CSF moieties in the fusion proteins was demonstrated by the result of clonogenic assay performed with human bone mononuclear, in which both SCF/M-CSF and M-CSF/SCF induced much higher number of CFU-M than equimolar amount of SCF or M-CSF or that of two cytokines mixture.

Introduction

Hematopoietic recovery of the patients such as those receiving bone marrow transplantation, anticancer radiotherapy and chemotherapy depends on the survival and the active proliferation/differentiation process of hematopoietic stem and progenitor cell, which require many cytokines especial early cytokines support. Single or late-acting hematopoietic growth factor therapy was proved to be not efficient enough to ensure hematopoiesis reconstitution [1]. Several hematopoietic cytokines were combined together through a link peptide to yield fusion protein with double function, such as IL-3/GM-CSF, Epo/IL-3, GM-CSF/EPO, etc [2–6]. Some of them had been so successfully designed that the functions of both moieties in fusion protein were enhanced, the limitation of each moiety was alleviated and even

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yielded new function because of their synergistic action. But there were other fusion proteins designed unsuccessfully and produced less activity than expected. One probably reason of this is the unsuitable link peptide or the unsuitable array order of the two moieties of the fusion protein, which may result in the spatial obstruction. So the study of the structures of cytokines and the correlated receptors is very important before designing the fusion protein.

Stem cell factor (SCF) stimulates the survival, proliferation, and differentiation of hematopoietic cells in combination with a lot of hematopoietic growth factor (HGFs) such as G-CSF, M-CSF, GM-CSF, EPO, TPO, IL-3, etc. It is clinically important for accelerating hematopoietic recovery in the treatment of myelodysplastic syndromes and after bone marrow transplantation [7–15]. SCF exists naturally as membrane-anchored and soluble isoforms as a result of alternative RNA splicing and proteolytic processing. The soluble form of SCF has 165 amino acids, but its receptor-binding core has been mapped to the first 141 residues [16–18]. SCF functions as a noncovalent homodimer, but under normal physiological conditions, the majority of soluble SCF exists as monomers. The biological activity of covalent dimeric SCF was about 10–20 folds higher than that of monomeric SCF with lower side effect [19–20]. For therapeutic purposes, the more potent disulfide-linked dimer is preferred because it can be administered at low doses to avoid significant mast cell activation while stimulating hematopoietic recovery. With the detailed structural information available, it may now be possible to design novel SCF variants with increased therapeutic potency.

M-CSF is another important hematopoietic growth factor that acts on hematopoiesis by promoting the growth of monocyte/macrophage colonies from human blood CD34+ progenitor cells and high proliferation colony-forming cells. It can also stimulate the macrophagic production of numerous hematopoietic cytokines (GM-CSF, G-CSF, IL-1, 6, 8, etc) [21–23]. In clinical trial, M-CSF can accelerate the recovery of the hematopoiesis directly or in synergy with other hematopoietic cytokines [21–23]. M-CSF can also potentiate the ability of mature mononuclear phagocytes to perform their differentiated functions by enhancing their ability to kill infectious microorganisms and tumor cells [24–26]. Under physiological conditions, the residue Cys31 forms the only interchain disulfide bond in M-CSF homodimer. It has been shown that the amino-(N-) terminal 149 amino acids of M-CSF are sufficient for its biological activity [27–28].

M-CSF and SCF can act in synergistic way to produce higher number of mononuclear phagocytes [29–31]. The receptors for SCF and M-CSF on target cells, c-kit and c-fms, are the members of the type III receptor tyrosine kinase subfamily. They are characterized by a common structure of the extracellular ligand binding domains that include five immunoglobulin Ig-like domains [32]. Former reports have shown that the second and third extracellular domains were involved in ligand recognition, while a putative dimerization site might be localized in the fourth extracellular domain. The first Ig-like domain, however, apparently imposed a small negative effect on binding of ligand [33].

Here we report the design of fusion protein SCF–M-CSF and their biological activities. First, several existing crystal structures were used as templates to generate models of the complexes of D1–D3 domains of receptor c-kit and c-fms with fusion protein SCF–M-CSF. The structure rationality of the fusion protein incorporated by a series of flexible linker peptide were analysed on InsightII system according to our previous work [34, 35]. Then, a suitable peptide was chosen for the fusion protein. Two recombinant fusion proteins were generated by construction of a plasmid in which the coding regions of human SCF (1–165aa) and M-CSF (1–149aa) cDNA were connected by this linker peptide coding sequence followed by subsequent expression in insect cell. The results of Western-blot and activity analysis showed that these two recombinant fusion proteins existed as a dimer form and had higher but different activity in stimulating the growth of SCF-dependent cell line, TF-1, and macrophage colony formation compared to SCF, M-CSF and SCF plus M-CSF.

Materials and methods

Restriction enzymes, Klenow fragment of DNA polymerase I and T4 DNA ligase were obtained from New England BioLabs (USA); [α-^{32}P]dATP

was obtained from DuPont/NEN (USA); Insect cell culture medium TNM-FH was from Sigma (USA); the pure recombinant human SCF was from New England BioLabs (USA); the pure recombinant human M-CSF expressed in silkworm was produced in our lab. Lipofectin was from Gibco BRL.

The Sf9 cell line was maintained in TNM-FH with 10% FBS. Transfer vector pVL1392 was bought from Invitrogen Corporation (USA).

Hematopoietic cell line TF-1 was grown in RPMI1640 supplemented with 10% fetal bovine serum and 3 ng/mL IL-3 or 5 ng/mL GM-CSF. Human leukemia cell line HL-60 was grown in RPMI1640 supplemented with 10% fetal bovine serum.

Molecular modeling of C-kit, C-fms 1–3 domains and the fusion protein complex

The sequences of the D1–D3 domains of c-kit, c-fms and the 142–165aa of SCF were extracted from the NCBI. The crystallographic structures of protein used as template were obtained from the PDB Bank (http://www.rcsb.org/pdb/). The constant domains of human Fc fragment (PDB file code 1FC1) were used as templates to construct the D1, D2, and D3 domains of c-kit. The fragment Fc-GAMMA receptor III complex (PDB file code 1E4K) and the hydrolase monoclinic form of *Trypanosoma cruzi* trans-sialidase (PDB file code 1MS1) were used as templates to construct D1, D2, D3 domains of c-fms. The crystal structure of a migration inhibitory factor-related rotein 8 (PDB file code 1MR8) and a kind of Oxidoreductase called methyl-coenzyme M reductase from *Methanopyrus kandleri* (PDB file code 1E6V) were used as templates to construct the model for the 142–165aa of SCF. Model building; docking experiments, energy minimization, and molecular dynamics studies were carried out using the software package InsightII (Molecular Simulation, Inc. 2000, 200 Fifth Avenue, Waltham, MA 02154) implemented on a Silicon Graphics Iris Indigo (SGI Inc., Silicon, CA, USA) workstation.

The fusion protein models were combined as head–tail form of SCF and M-CSF using a series of linked peptide of different length and were verified finally with the biopolymer module implemented in insightII. The models of the complexes of receptor D1–D3 domain with fusion protein were constructed as follow. The structure of domain D2 of receptor Flt-1 in complex with VEGF (PDB file code 1QTY_S and 1QTY_R) was used as a template for ligands interactions with receptors according to the previous paper [36]. Briefly, keeping the dyad symmetric receptor pair fixed, VEGF was replaced with SCF or M-CSF. Then, domain D2 of receptor Flt-1 was replaced with D1–D3 domain of receptor c-kit or c-fms to form the ligand–receptor complex. The geometry of the protein was optimized in the same way as above. Finally, fusion protein was superimposed SCF or M-CSF. Since the interaction sites of SCF or M-CSF can been obtained in the reference [37–39], we labeled them in the model of receptor–cytokine complexes, manual rotations of the fusion protein and receptor were carried out to avoid unfavorable interactions and form proper complexes of fusion protein with receptor D1–D3 domains.

The energy minimization procedure consisted of 1000 steps of steepest descent, and then a conjugate gradient minimization was taken until the root mean square (rms) gradient of the potential energy was less than 0.001 kcal/molA. The minimized coordinates of the protein, receptor, and complex were used as starting point for dynamics. During dynamics, the lengths of the bonds involving hydrogen atoms were constrained according to the SHAKE algorithm, which allows an integration time step of 0.001 picoseconds (ps).

The complexes of receptor with cytokine or the complexes of receptor with fusion protein were thermalized to 300 K with a gradient of 5 K per 6000 steps by randomly assigning individual velocities from the Gaussian distribution. After heating, the systems were allowed to equilibrate until the potential energy vs. time was approximately stable (34 ps). Velocities were scaled by a single factor. An additional 10-ps period of equilibration with no external perturbation was taken. Time-averaged structures were then determined over the last 100 ps of each simulation for the hormones and the last 40 ps of each simulation for the complexes. Data were collected every 0.5 ps.

The most suitable link-peptide was picked out by analyzing the intermolecular energy between second moieties in fusion protein and D1 domain in receptor-fusion complexes. The energies of intermolecular interaction were calculated under

the Docking module of insightII. The second moiety was defined as a subset in the fusion protein, and then the main energy was calculated as intermolecular interaction in the Docking module. Among the results, the link-peptide that produced the lowest intermolecular energy was picked out as the most suitable one for further fusion protein gene construction.

Expression of rhM-CSF/SCF and rhSCF/M-CSF

PCR was performed to construct recombinant transfer vector carrying the SCF/M-CSF and M-CSF/SCF cDNA. The DNA fragment encoding natural SCF signal peptide (25aa), SCF 1–165aa and the first part of the linker sequence (GGGGS) was obtained from pUC18–SCF by PCR performed with a sense primer (5′-GGTCTAGA-TGAAGAAGACACAAACT-3′) and an antisense primer (5′-CCGGATCCTCCTCCTCCGGCTG-CAACAGGGGGTAA-3′). The PCR product was double digested with *Xba*I and *Bam*HI, purified by agarose gel electrophoresis, and cloned into pUC18 to yield pUC18–5′SCF. The DNA fragment encoding the second part of the linker sequence (GSGGGGSGG), truncated M-CSF (1–149aa) and double terminal codon (UAAUAG) was obtained from pUC18–M-CSF cDNA plasmid by PCR performed under the similar conditions with a sense primer (5′-GGGGATCCGGA GGAGGAGGCTCCGGCGGCAGTATCACCG-AGG AGGTGTCG-3′) and an antisense primer (5′-GCGGTACCCGGGCTATTATTGGCTGGA-GCA TTC-3′). After being double digested by *Bam*HI and *Kpn*I, the purified 3′-M-CSF fragment was cloned into pUC18–5′SCF to yield pUC18–SCF/M-CSF. pUC18–M-CSF/SCF was constructed in a similar way.

The M-CSF/SCF and SCF/M-CSF cDNA fragment were individually inserted into baculovirus transfer vector pVL1392. The recombinant transfer vector and AcNPV DNA were co-transfected into Sf9 cells using Lipofectin. After three rounds of plaque purification, the polyhedrin-negative viruses were isolated. The recombinant virus with the highest biological activity was chosen for further study.

Sf9 cells (3×10^6/25 cm^2 flask) were seeded into flasks. After attachment of the cells, the medium was removed and the cells were infected with recombinant virus at MOI of 10. After 1 h, the medium was replaced by 5 mL of fresh TMN-FH medium without FBS. After 72 h incubation at 27 °C, the culture supernatant was collected for further activity assay. Quantitation of fusion protein was determined by M-CSF ELISA assay, using mAb against M-CSF.

Analysis of expressed proteins

The express protein was analyzed by Western blot under both reducing and non-reducing conditions. The samples were separated by SDS-PAGE and were electrophretically transferred onto nitrocellulose membrane. Immunoblot analysis was carried out according to the protocol recommended by Bio-Rad. Rabbit polyclonal antibodies against human SCF and M-CSF were used as the primary antibody respectively and horseradish peroxidase-conjugated anti-rabbit IgG antibody as the detecting reagent.

Activity assays

Cell proliferation assays were conducted on both human leukemia cell line TF-1 expressing SCF receptor c-kit but not M-CSF receptor c-fms and human acute promyloid cell line HL-60 expressing M-CSF receptor but not SCF receptor.

The TF-1 cell proliferation activity assay for the SCF moiety of fusion protein was performed as described [40]. Increasing amounts of pure rhSCF and recombinant SCF/M-CSF, M-CSF/SCF diluted with RPMI1640 were made in 96-well microtiter plates. TF-1 cells at final concentration of 5×10^4/mL per well were added at time zero. After 48 h incubation at 37 °C in fully humidified atmosphere containing 5% CO_2, the growth of TF-1 cell was determined by the colorimetric MTT method [41]. The HL-60 cell proliferation activity assay for the M-CSF moiety of fusion protein was performed as described [42]. Increasing amounts of pure rhM-CSF and rhSCF/M-CSF, rhM-CSF/SCF diluted with RPMI1640 were made in 96-well microtiter plates. HL-60 cell at final concentration of 2×10^4/mL per well were added at time zero. The follow steps were the same as those of the SCF activity assay.

Human mononuclear cells (MNC) were isolated from health donor's bone marrow cells using Ficoll-Hypaque (LYMPHOPREP, 1.077 ± 0.001 g/mL; Nycomed, Oslo, Norway) density gradient

centrifugation. Soft agar Macrophages colony-forming assays were performed as previously reported [43]. Colonies that consisted of more than 50 cells were counted after incubating for 7 days or longer in a fully humidified atmosphere containing 5% CO_2.

Results

Modeling of C-kit and C-fms 1–3 domain and fusion protein complex

The complete models of complexes of receptor D1–D3 domains with SCF–M-CSF fusion proteins were built by using the Ca coordinates of M-CSF, SCF and the constructed receptors. Then they were subjected to a 150 ps dynamic run. The average structures obtained, computed on the last 100 ps were further minimized. The minimization stage and different kinds and strength of constraints in both minimization and dynamics were judged on the basis of the root mean square (rms) deviation from the crystal structure and computational efficiency.

The structure module of D1–D3 domains of c-kit and c-fms and fusion protein SCF/M-CSF or M-CSF/SCF were successfully constructed on insightII. The structures of D1–D3 domains of c-kit and c-fms present three connected absolute domains construction just like the reported Ig-like domain. The module of fusion protein SCF/M-CSF presents cross-like structure, and the module of fusion protein M-CSF/SCF presents parallel-like structure. The structure modules of fusion proteins with 12aa link-peptide were shown in Figure 1.

Choice of suitable link-peptide

Although the energy of total system was under zero, the interaction between the second moiety in fusion protein and receptor D1 domain really existed. The maximal partial intermolecular actions between the second moiety in fusion protein with different link peptide and two receptor D1 domains are listed in Table 1. It was obviously that the SCF moiety of M-CSF/SCF couldn't interact with D1 of the c-fms, and the M-CSF moiety of SCF/M-CSF with a 12aa connecting peptide produced the lowest energy with D1 of both C-kit and C-fms. So the peptide Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly was used as link peptide to connect the two moieties of fusion protein. The module of interaction between c-kit D1–D3 domain and SCF/M-CSF (12aa link peptide) was shown in Figure 2a, and the module of interaction between c-fms D1–D3 domain and M-CSF/SCF (12aa link peptide) was shown in Figure 2b.

The characterization and bioactivity of rhM-CSF/SCF and rhSCF/M-CSF

Recombinant M-CSF/SCF or SCF/M-CSF could be expressed in the Sf9 cells infected with the

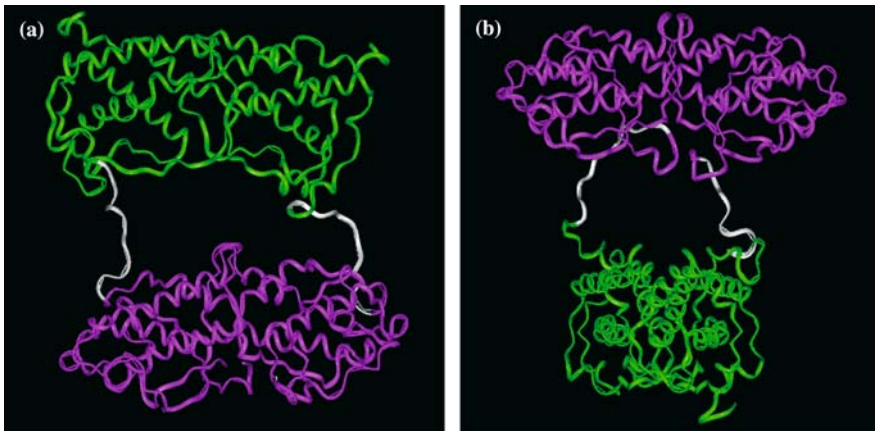

Figure 1. The structure modules of M-CSF/SCF (a) and SCF/M-CSF (b) with 12aa linker peptide. (The green stands for M-CSF, the mauve stands for SCF and the white stands for linker peptide).

Table 1. The link-peptides and the intermolecular action values (expressed as Van der Waals) produced by the second moiety in fusion proteins with different link peptide to D1 domains of receptor c-kit and c-fms.

		Intermolecular action of the second moiety to D1 domain of receptor (Van der Waals)	
		Receptor	
Fusion protein	Link peptide sequence	M-CSF moiety to c-kit	SCF moiety to c-fms
SCF/M-CSF	GGGGSGGGGSGG	< 1.673e + 9	< 1.063e + 13
	GGGSGGGGSG	< 6.969e + 11	< 1.144e + 18
	GGSGGGSG	< 2.113e + 12	< 4.161e + 18
	GGGG	< 1.694e + 15	< 2.2688e + 18
M-CSF/SCF	GGGGSGGGGSGG	> 1.000e + 20	< 0
	GGGSGGGGSG		
	GGSGGGSG		
	GGGG		

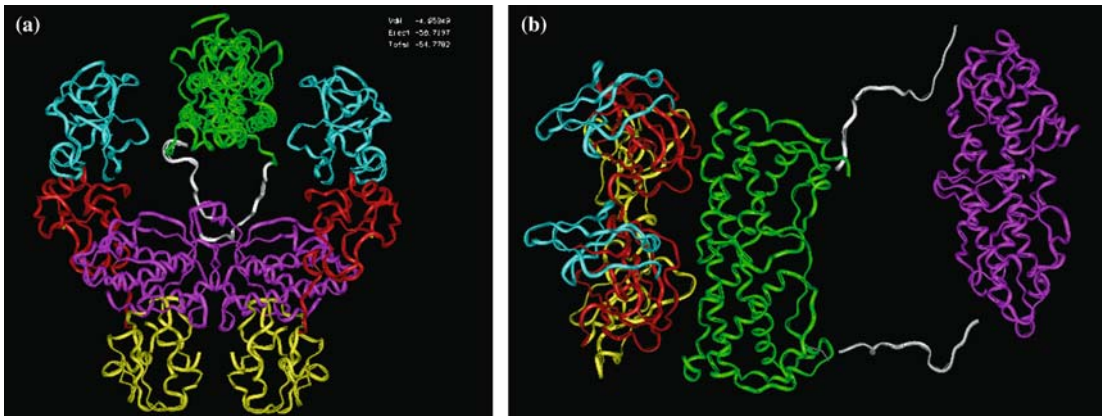

Figure 2. The module of intermolecular action between fusion protein and receptor. (a) SCF/M-CSF and C-kit D1-D3 domains complex (Green: M-CSF, mauve: SCF, bluish: D1 domain, red: D2 domain, yellow: D3 domain) and (b) M-CSF/SCF and C-fms D1-D3 domains complex (Green: M-CSF, mauve: SCF, bluish: D1 domain, red: D2 domain, yellow: D3 domain).

recombinant virus AcNPV-M-CSF/SCF or AcNPV SCF/M-CSF. Neither M-CSF activity nor SCF activity was detected in the medium of Sf9 cells infected with wt-AcNPV.

SDS-PAGE and Western Blot analysis showed that both rhM-CSF/SCF and rhSCF/M-CSF existed as dimer with a molecular weight of ~84 KD under non-reducing condition, and monomer of ~42 KD under reducing condition (Figure 3).

The biological activities of SCF or M-CSF moiety in each fusion protein were evaluated by their abilities to support the growth of TF-1 expressing SCF receptor but not M-CSF receptor and HL-60 cell lines expressing M-CSF receptor but not SCF receptor in proliferation assays. Results showed that each fusion protein induced a dose-dependent proliferative response on each cell lines. In SCF activity assay with TF-1 cell line, the ED_{50} for SCF/M-CSF and M-CSF/SCF was 1 ng/mL and 2.4 ng/mL (12 pmol/L and 28.6 pmol/L based on a predicted molecular mass of 84 000 Daltons,) respectively, while the ED_{50} for monomer SCF was 5.2 ng/mL (280 pmol/L based on a predicted molecular mass of 18 000 Daltons). Thus, SCF/M-CSF was 20-fold more potent than an equimolar of standard SCF while M-CSF/SCF had about 10-fold activity of SCF (Figure 4). In M-CSF activity assay with HL-60 cell line, the ED_{50} for SCF/M-CSF and M-CSF/SCF was 11 ng/mL, while the ED_{50} for M-CSF was about 6 ng/mL. Thus, no apparently difference was found between the fusion proteins and M-CSF in terms of M-CSF activity (Figure 5).

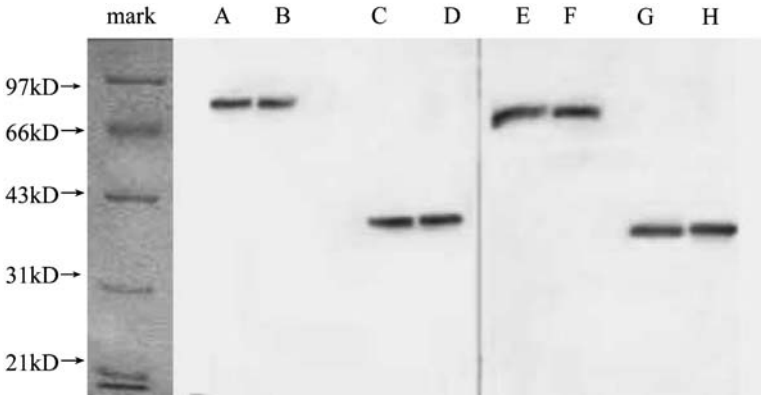

Figure 3. Characterization of SCF/M-CSF and M-CSF/SCF fusion protein. The SCF/M-CSF and M-CSF/SCF fusion protein were separated on 12% SDS-PAGE, and then transferred to nitrocellulose and stained (1) with a mAb to human M-CSF (lane A for SCF/M-CSF, lane B for M-CSF/SCF at non-reducing condition and lane C for SCF/M-CSF, lane D for M-CSF/SCF at reducing condition), and (2) with mAb to human SCF (lane E for SCF/M-CSF, lane F for M-CSF/SCF at non-reducing condition and lane G for SCF/M-CSF, lane H for M-CSF/SCF at reducing condition).

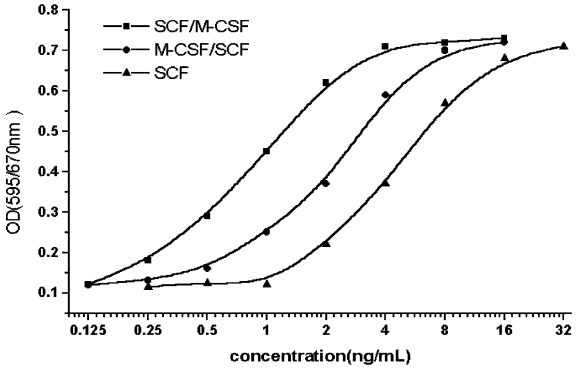

Figure 4. Effects of rhSCF/M-CSF and *E. coli*-derived monomeric rhSCF on TF-1 cells proliferation. The TF-1 cells were incubated with various concentrations of the rhSCF/M-CSF (■), rhM-CSF/SCF(●) and rhSCF (▲). After incubation, the activity was assayed as described in Materials and Methods. The ED_{50} of rhSCF/M-CSF and *E. coli*-derived rhSCF were 13 pmol/L (1.09 ng/mL) and 230 pmol/L (4.1 ng/mL), respectively.

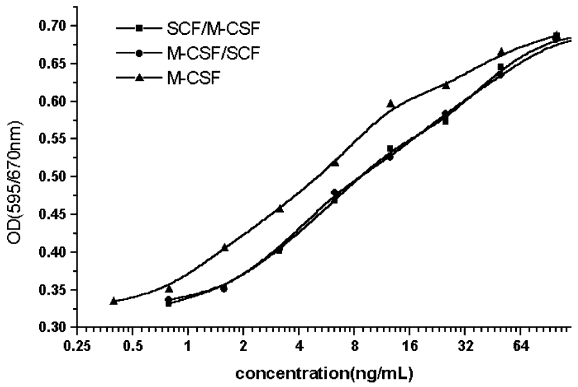

Figure 5. Effects of rhSCF/M-CSF and rhM-CSF on HL-60 cells proliferation. The HL-60 cells were incubated with various concentrations of the rhSCF/M-CSF (■), rhM-CSF/SCF(●) and rhM-CSF (▲) as described in Materials and Methods.

The biological activity of the each fusion protein was also evaluated in clonogenic assays performed with human bone mononuclear. The results indicated that, at each concentration group, both SCF/M-CSF and M-CSF/SCF induced much higher number of CFU-M compared to the SCF and M-CSF alone or in combination. (Figure 6).

Discussion

Large dose uses of SCF in clinic often cause dermal mast cell degranulation induced allergic-like reaction [44–45], while long time and high dose uses of M-CSF will cause cellular or organ damage and inhibition of hematopoiesis [46–47]. We designed the M-CSF–SCF fusion protein that could form homodimer through Cys31–Cys31 in M-CSF subunit to increase the activity of SCF subunit in the fusion protein. This may also raise M-CSF subunit activity of the fusion protein through synergistic action between SCF and M-CSF. So that, clinic dose of SCF and M-CSF can be decreased, and less side effect will be produced. It should be noticed that different to the normal receptor–ligand reactions, D1 domains of the receptors might produce spatial encumbrance to the binding of fusion protein because of the

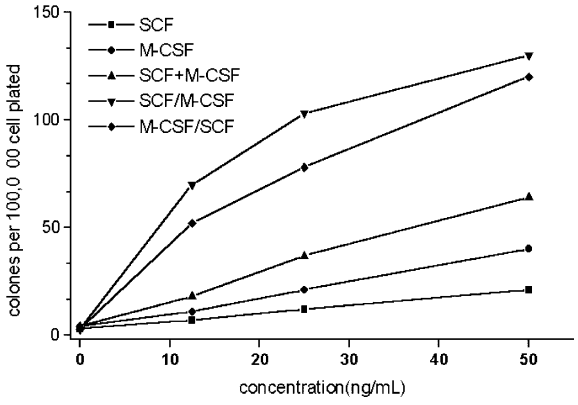

Figure 6. Effects of rhSCF/M-CSF and rhM-CSF/SCF on macrophage-colony forming. Human bone marrow mononuclear cells were isolated and grown as described in Materials and Methods. Equimolar rhSCF/M-CSF and rhM-CSF/SCF, *E.coli*-derived monomeric rhSCF, rhM-CSF and mixture of monomeric rhSCF and rhM-CSF (1:1) were assayed respectively at several concentrations.

existing of second moiety in fusion protein. As no three-dimensional structural information is available for the SCF-c-kit complex, the design of M-CSF–SCF fusion protein was operated on the computer using Insight II software at first. Previous researches showed that dimeric SCF formed a complex with two molecules of the extracellular domain of its receptor [48], we constructed the models of domains D1–D3 of receptors c-kit or c-fms in complex with dimeric SCF or M-CSF using three-dimensional structure of domain D2 of receptor Flt-1 in complex with VEGF as a template, because receptor Flt-1 belong to the same type III tyrosine kinase receptors characterized by the conservative extracellular ligand binding domains including five immunoglobulin Ig-like domains. Then the fusion proteins were superimposed to the cytokine in the models. A series of flexible linker sequences were incorporated into the fusion protein and then their structure rationalities were analysed. The docking analysis indicated that a flexible linker sequence (GGGGSGGGGSGG) might be the best. The activity assays using TF-1 cell line expressing SCF receptor but not M-CSF receptor and HL-60 cell lines expressing M-CSF receptor but not SCF receptor showed that fusion protein could promote the a dose-dependent proliferative response on both cell lines. So the linker peptide was flexible enough for the two moieties in the fusion protein to interact with binding sites in the receptor.

SCF and M-CSF dimerize in various ways. Dimerization of M-CSF is dictated by the position of the Cys31–Cys31′ intermolecular disulfide bond, while intermolecular hydrogen bond pairs in SCF, like other noncovalent dimeric protein such as Flt-3 ligand and IL-8 [39, 49–50], replace the intermolecular disulfide bond between the two M-CSF protomers. It was reported that the disulfide-linked SCF (A165C) would be 100% dimer in associated form at all concentrations and had a 10-fold higher biological activity than wild type rhSCF [51]. The result of western blot indicated that the fusion proteins could form homodimmer through Cys31–Cys31 in M-CSF subunit in the fusion protein, so the SCF moieties could also form the dimmer just like the condition of the disulfide-linked SCF (A165C). It was demonstrated by the results of cell proliferation assays of two fusion protein on TF-1 cell line which showed that the SCF activity of them were much higher than that of the monomic form SCF at equimolar. The enhanced synergistic effects of SCF and M-CSF moieties in fusion proteins were demonstrated by colony-forming assay in which the colony-forming capacities induced by fusion were much higher than that of M-CSF, SCF alone or M-CSF plus SCF. This may be caused by dimeric former of SCF moieties.

However, two fusion proteins had the different activity either in promoting cell proliferation or in colony-forming capacity. Although the M-CSF activities of two fusion proteins were nearly the same in promoting the HL-60 cell proliferation, the degrees of promoting the TF-1 cell proliferation and colony forming capacity induced by the two fusion protein were different. The rhSCF/M-CSF had higher activity than rhM-CSF/SCF. This might be due to the different array of two moieties in fusion proteins. Structural study of SCF had shown that the initial 1∼4 amino acids on N-terminal region of SCF partly took part in binding to its receptor and were very important for its activity [37–39]. So the N-terminal M-CSF moiety of M-CSF/SCF may partly affect the C-terminal SCF moiety's binding to its receptor. Meanwhile, as the binding sites of M-CSF to its receptor consisting of His9, His15 and other amino acid were on the same side face of M-CSF dimer [52], the SCF moiety on either SCF/M-CSF or M-CSF/SCF did not affect M-CSF moiety's binding to its receptor. It could also be surmised that the increased

potential of fusion protein in the stimulating CFU-M, compared to that of the mixture of SCF and M-CSF, could most likely attribute to the dimerization of SCF moieties in the fusion protein. The results of docking analysis of the computer models were consistent with such biological activity assays. Other fusion protein also showed such different activity of two moiety caused by their different array on fusion protein and by different kind/length of link peptide.

Conclusion

The ligands for each of these receptors can exist as dimers, non-covalently associated in the cases of SCF and disulfide-linked in the cases of M-CSF, and ligand dimerization mediates receptor dimerization. The molecular models of the complexes of fusion proteins and their receptor's D1–D3 domains have been constructed successfully using the software Insight II, while the docking analysis helped in picking out the most suitable connecting peptide, which has been verified by the biological activity assays. It is believed that the computer aid design is fairly suggestive of being adopted in studies that concern construction of hematopoietic fusion proteins, especially those including SCF moiety.

Acknowledgements

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