Pharmaceutical Research, Vol. 23, No. 9, September 2006 (© 2006)
DOI: 10.1007/s11095-006-9059-5

Research Paper

Improving the Oral Efficacy of Recombinant Granulocyte Colony-Stimulating Factor and Transferrin Fusion Protein by Spacer Optimization

Yun Bai[1] and Wei-Chiang Shen[1,2,3]

Received April 8, 2006; accepted May 8, 2006; published online August 9, 2006

Purpose. To improve the oral efficacy of the recombinant fusion protein containing granulocyte colony-stimulating factor (G-CSF) and transferrin (Tf) by inserting a linker between the two protein domains.
Materials and Methods. Oligonucleotides encoding flexible and helix-forming peptides were inserted to the recombinant plasmids. The fusion protein without linker insertion was used for comparison. The G-CSF cell-proliferation and Tf receptor-binding activities of the fusion proteins were tested in NFS-60 cells and Caco-2 cells, respectively, and *in vivo* myelopoietic assay with both subcutaneous and oral administration was performed in BDF1 mice.
Results. All fusion proteins produced from transfected HEK293 cells were positive in Western-blotting assay with anti-G-CSF and anti-Tf antibodies. Among them, the fusion protein with a long helical (H4-2) linker showed the highest activity in NFS-60 cell proliferation assay, with an EC_{50} about ten-fold lower than that of the non-linker fusion protein. The fusion protein with H4-2 linker also showed a significantly higher myelopoietic effect when administered either subcutaneously or orally in BDF1 mice.
Conclusion. The insertion of a linker peptide, such as the helix linker H4-2, between G-CSF and Tf domains in the recombinant fusion protein can improve significantly both *in vitro* and *in vivo* myelopoietic activity over the non-linker fusion protein.

KEY WORDS: G-CSF; linker peptides; oral delivery; recombinant fusion protein; transferrin.

INTRODUCTION

Recombinant proteins are becoming an important class of therapeutic drugs (1,2). Many recombinant proteins such as growth hormones and humanized monoclonal antibodies are already in clinical use (3,4). Most peptide and protein drugs are limited to injection in their administration due to their size, charge and hydrophilicity. Furthermore, the sensitivity of fusion proteins to digestive enzymes also imposes a difficulty for oral delivery. However, frequent injections can cause the inconvenience, poor compliance, and adverse side-effect of the protein drugs to patients. Therefore, oral administration is still the most desirable route for the development of protein drug delivery systems (5,6). One approach for the oral delivery of protein drugs is to explore the receptor-mediated transcytosis in intestinal epithelia (7).

Previous studies have demonstrated that transferrin receptor (TfR) can be used for increasing epithelial absorption of protein and peptide drugs (8–13). We have shown that Tf and its conjugates can be transported across various cultured epithelial cell monolayers, including MDCK (14), Caco-2 (15), and primary rat alveolar epithelial cells (16). Furthermore, we have reported oral hypoglycemic activity of an insulin-Tf conjugate in diabetic rats (17,18), and oral myelopoietic activity of a G-CSF and Tf conjugate in BDF-1 mice (19). Most recently, we have produced a fusion protein from recombinant cDNA of G-CSF (20 kD) and Tf (80 kD) that possessed both transferrin receptor (TfR) binding ability in Caco-2 cells and the proliferative activity in NFS-60 cells (20). Furthermore, we have demonstrated that the G-CSF-Tf fusion protein can be absorbed across gastrointestinal epithelia, as indicated by its myelopoietic activity when orally administered in BDF-1 mice (20). However, the fusion protein only retained a small fraction of the *in vitro* activity of both cell proliferation of G-CSF and TfR-binding of Tf. Conceivably, the low *in vitro* activity may be an indication of the interference of the two domains with each other in the conformation of the fusion protein. Such interference will most likely decrease the binding of the fusion protein to G-

[1] Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90033, USA.
[2] PSC 404B,1985 Zonal Avenue, Los Angeles, California 90033, USA.
[3] To whom correspondence should be addressed. (e-mail: weishen@usc.edu)

ABBREVIATIONS: ANC, absolute neutrophil count; G-CSF, granulocyte colony stimulating factor; G-CSF-Tf, recombinant fusion protein consisting of G-CSF and Tf; G-CSF-LE-Tf, the fusion protein of G-CSF and Tf with a LE dipeptide linker; GS, linker peptide with the amino acid sequence of (GGGGS); H2, linker peptide with the amino acid sequence of $A(EAAAK)_2A$; H3, linker peptide of $A(EAAAK)_3A$; H4, linker peptide of $A(EAAAK)_4A$; H4-2, linker peptide of $A(EAAAK)_4A$; Tf, transferrin; TfR, transferrin receptor.

CSF and Tf receptors and, consequently, the *in vivo* myelopoietic effect in the animal studies.

In order to optimize the intrinsic bioactivity of the fusion protein, we investigate the insertion of spacers between G-CSF and Tf domains that will keep them at a distance. Several studies have indicated that the flexibility and hydrophilicity of the peptide linkers are important for the preservation of the functions of the individual domains in a fusion protein (21,22). The flexible linker, $(GGGGS)_3$, is the most commonly used linker between fusion protein domains, especially for single-chain Fv, sFv, which consists of V_L and V_H domains of the immunoglobulin (23). However, it has been reported that other fusion proteins may lose their activity using the flexible spacer to link the two moieties (24). Furthermore, for orally administered proteins, a flexible linker without secondary structures will be easily accessed by the digestive enzyme in the GI tract. More recently, a peptide linker, $A(EAAAK)_nA$, has been reported to form an α-helix conformation, which could control the distance and reduce the interference between the domains (25). It was reported that the helix linker could effectively separate bifunctional domains of the fusion protein (26,27).

In this paper, five G-CSF-Tf fusion proteins with different linkers were developed and compared with the fusion protein without the inserted linker. The fusion protein that showed the highest bioactivity in the *in vitro* studies also exhibited an improved myelopoietic efficacy for both subcutaneous injection and oral administration in BDF-1 mice.

MATERIALS AND METHODS

Plasmid Construction

The method for plasmid construction was similar to that as described in the previous report (20). Human G-CSF cDNA harboring the signal peptide was cloned from human bladder carcinoma 5637 (American Type Culture Collection) by using RT-PCR. Human Tf cDNA was subcloned from the plasmid TFR27A (American Type Culture Collection). Expression plasmid containing G-CSF fused in frame with Tf was engineered by using the mammalian expression vector pcDNA3.0. A dipeptide linker, Leu-Glu, was introduced between the G-CSF and Tf as a short connection. The sequence was confirmed by DNA sequence analysis.

The Insertion of Peptide Spacers into the G-CSF-Tf Plasmid

Annealed synthetic phosphorylated oligonucleotides were used to create the spacers, consisting of double strand DNA between G-CSF and Tf. The spacers were designed with sticky ends that were complimentary with the *Xho 1* cutting site. The oligonucleotides were dissolved in Tris-EDTA buffer to a final concentration of 20 pmol/μl. To 1 μl of each oligonucleotide solution (both forward and reverse sequences), 2 μl (10×) annealing buffer (100 mM Tris HCl, pH 7.5, 1 M NaCl, 10 mM EDTA) was mixed and ddH_2O was added to make a final volume of 20 μl. The mixture was heated to 95°C for 10 min, and allowed to cool down gradually to room temperature to form the double-stranded DNA with 5′-overhangs that were complimentary to the *Xho 1* cutting site. The double-stranded DNA spacers were ligated to the *Xho 1* cut G-CSF-Tf plasmid that had been treated with phosphatase (New England Biolabs). The spacer-vector ratio and the ligation temperature were adjusted to control the copies of spacer inserted. A 5 μl aliquot of the ligation mixture was used to transform JM109 competent cells. The DNA sequence of each spacer-containing plasmid was determined and verified.

Expression of Fusion Proteins and Western-Blot

HEK293 cell monolayers were transfected with different plasmids by using Lipofectamine 2000 (Invitrogen). After a 5 h incubation, the protein free medium CD293 of the cultured HEK293 cells was replaced with the same medium. After a five-day culture, the conditioned medium from each plasmid-transfected HEK293 cell culture was collected and subjected to 10% SDS/PAGE analysis. Western blotting analysis was performed by using anti-G-CSF and anti-Tf antibodies. Horseradish peroxidase activity was detected by the enhanced chemiluminescence method.

The G-CSF-Dependent NFS-60 Cell Proliferation

The G-CSF activity of the fusion protein was measured by NFS-60 cell proliferation assay (28,29). NFS-60 is an interleukin-3/G-CSF-dependent murine myeloid leukemia cell line. NFS-60 cells were washed three times with RPMI-1640/10% FBS and aliquoted into 96-wells microtiter plates at a seeding density of 1×10^5 cells/ml. Subsequently, 10 μl of ten-fold serial dilutions of the G-CSF and fusion proteins was added to each well. The plate was incubated at 37°C in a 5% CO_2 incubator for 48 h. A MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed. Briefly, the cells were treated with 1 mg/ml MTT in serum-free and phenol red-free RPMI 1640 media for 4 h. The formazan crystals were formed and were dissolved in isopropanol. The absorbance of the formazan solution was measured at 570 nm on a TECAN GENios Plus microplate reader.

TfR Binding Assay

Human Tf was radiolabeled with ^{125}I (ICN, Irvine, CA) using the chloramine-T catalyzed iodination method (30). The iodinated Tf was purified first by using Sephadex G-50 column chromatography. The purified ^{125}I-Tf was subsequently dialyzed in 1,000× volume of phosphate buffered saline (PBS, pH 7.8) at 4°C for 18 h. Caco-2 cells were seeded in 12-well cluster plates and were cultured for 2 weeks until fully differentiated. Caco-2 monolayers were washed with cold PBS three times, and then incubated in serum-free D-MEM supplemented with 0.1% BSA at 37°C for 30 min to remove the endogenous Tf. A mixture of 3 μg/ml ^{125}I-Tf with 3-, 10- or 30-fold of unlabeled fusion proteins in serum-free D-MEM supplemented with 0.1% BSA was added to different wells. After 30 min of incubation at 4°C, the medium was removed, and the cell monolayer was washed with cold PBS three times. The cells were then dissolved in 1 M NaOH, and the lysates were counted in a gamma counter.

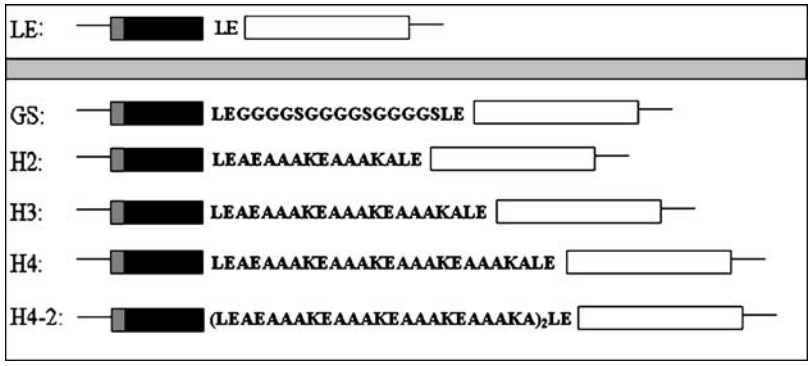

Fig. 1. The five fusion constructs, in comparison with the original no-linker construct. The intergenic linkages are shown. The *small grey box* at the N-terminal represents the secretion signal sequence; the *black box* represents G-CSF sequence; the *open box* represents Tf sequence.

The *In Vivo* Myelopoietic Activity in Mice

Male BDF1 mice (Charles River Laboratories, Wilmington, MA), 6–8 weeks of age, were used in the animal experiments. Animal experiments were compliant with the 'Principles of Laboratory Animal Care' (NIH Publication #85-23) and had been approved by the Institutional Animal Care and Utilization Committee at the University of Southern California. The mice were allowed to acclimate for 5 days before the experiment. Prior to the treatment, the mice were fasted for 12 h. The treatment groups received a single dose on day 0. Due to the difference in molecular weight, the dosage of each protein was based on equivalent micromoles.

Blood samples were collected daily from the tail vein of the mice, diluted 40-fold with PBS, and lysed in an acidic crystal-violet solution (0.1% crystal violet, 1% acetic acid, in water). The total white blood cell (WBC) count was determined manually with a hemacytometer. The percentage of polymorphonuclear neutrophil count, or absolute neutrophil count (ANC), was determined by multiplying the total WBC count by the percentage of PMN in each sample and was presented as number of neutrophils per microliter of blood.

RESULTS

Construction of Fusion Protein Plasmids and Fusion Protein Expression

The five fusion proteins with various spacer peptides, i.e., G-CSF-GS-Tf, G-CSF-H2-Tf, G-CSF-H3-Tf, G-CSF-H4-Tf, and G-CSF-$(H4)_2$-Tf, were all developed and expressed in cultured HEK293 cells (Fig. 1). After transfection, HEK293 cells were cultured in protein-free CD293 medium for 5 days. The five fusion proteins were detected by performing Western-blotting analysis of the collected conditioned medium from different transfected HEK293 cells. Results showed that all five fusion proteins could be recognized by anti-Tf antibody (Fig. 2a) and anti-G-CSF antibody (Fig. 2b). The increase of molecular weight of the five fusion proteins correlated well with the size of the linker peptides, from the smallest H2, 1.55 kD, to the largest H4-2, 4.8 kD (Fig. 2).

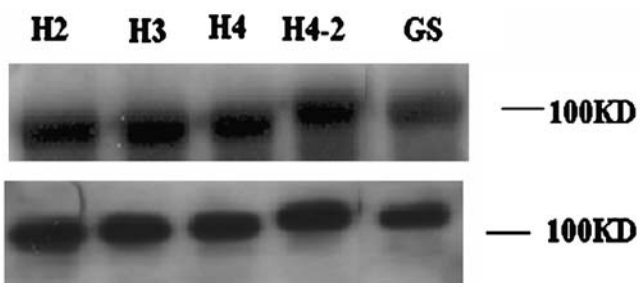

Fig. 2. The Western-blot result. (a) The five fusion proteins are recognized by anti-Tf antibody. (b) The five fusion protein are recognized by anti-G-CSF antibody.

In Vitro G-CSF Activity of the Fusion Proteins

The purified fusion proteins were assayed for G-CSF activity by measuring their ability to stimulate NFS-60 cell proliferation. Different amount of fusion proteins, which were sterile-filtered and normalized as G-CSF-equivalent, were included in NFS-60 cell culture medium to replace G-CSF as a cell growth factor. The EC_{50} was estimated by fitting the data with a sigmoidal curve (Table I). Fusion protein with H4-2 linker, i.e., G-CSF-$(H4)_2$-Tf, showed the highest cell proliferative activity, with an EC_{50} of 0.143 ng/ml. Therefore, G-CSF-$(H4)_2$-Tf was chosen to compare with G-CSF-LE-Tf fusion protein and the native G-CSF in NFS-60 proliferation assays. Fig. 3(a) showed that the activity of G-CSF-$(H4)_2$-Tf was about ten-fold higher than that of G-CSF-LE-Tf, but was still about less than 50% of that of the native G-CSF.

In Vitro Tf Receptor Binding Activity of the Fusion Proteins

As shown in Fig. 3(b), the addition of unlabeled fusion protein caused a decrease in the binding of ^{125}I-labeled Tf to TfR in cultured Caco-2 cells, indicating that the fusion protein maintained specific binding ability to TfR. However, both G-CSF-$(H4)_2$-Tf and G-CSF-LE-Tf had a similar binding affinity to TfR, which was only about 1/16th of that of native Tf.

In Vivo Characterization of the Fusion Proteins by Subcutaneous Injection

To investigate whether the *in vivo* myelopoietic efficacy of the fusion protein correlated with the *in vitro* biological

Table I. The EC_{50} of the Fusion Proteins in the NFS60 Proliferation Assay

Linker peptides in the fusion protein	EC_{50} (ng/ml)
GS, $(GGGGS)_3$	0.20
H2, $A(EAAAK)_2A$	0.19
H3, $A(EAAAK)_3A$	0.17
H4, $A(EAAAK)_4A$	0.20
H4-2, $A(EAAAK)_4ALEA(EAAAK)_4A$	0.14
LE	2.34

activity, 0.1 mg/kg (0.5 μmol/kg), 0.5 mg/kg (2.5 μmol/kg) and 1 mg/kg (5 μmol/kg) of G-CSF, as well as molar-equivalent amounts of G-CSF-$(H4)_2$-Tf or G-CSF-LE-Tf, were subcutaneously injected to BDF1 mice. The molecular mass of the fusion protein is approximately five times that of G-CSF itself (G-CSF is 20 kDa, whereas the fusion protein is about 100 kDa). The day of administration was denoted as day 0. By comparing the peak effect at 24 h, G-CSF and G-CSF-LE-Tf have similar myelopoietic effect at doses of 0.5 mg/kg and

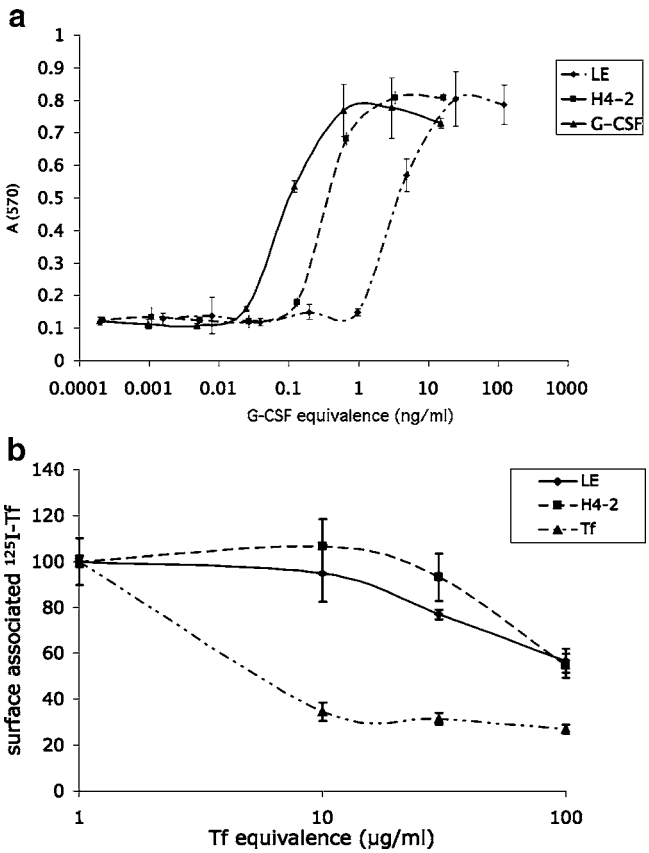

Fig. 3. *In vitro* characterization of the fusion proteins G-CSF-LE-Tf (LE) and G-CSF-$(H4)_2$-Tf (H4-2). (a) Evaluation of G-CSF activity of the purified fusion proteins. Proliferation of the murine myeloblastic cell line NFS-60 was measured via MTT assay. The concentrations of the fusion proteins were expressed as the G-CSF equivalence. *Error bars* represent SD, n=4. (b) The TfR binding activity of the fusion proteins on Caco-2 cells, with recombinant Tf as positive control. ^{125}I-labeled Tf (3 μg/ml in serum-free medium with 1 mg/ml BSA) was added to the medium of Caco-2 monolayers. Different concentrations of unlabeled fusion proteins were added to compete for TfR binding. *Error bars* represent SD, n=4.

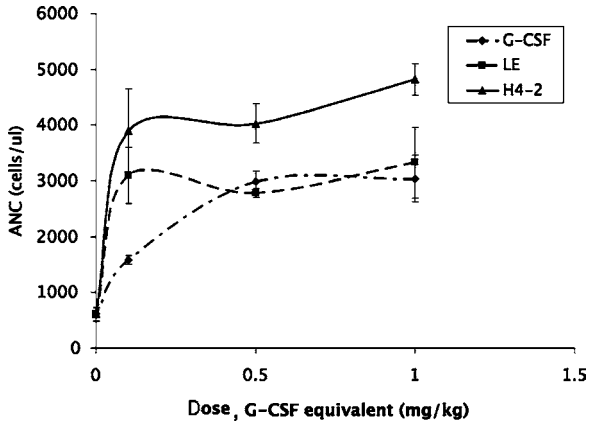

Fig. 4. The dose-response curve of subcutaneously administered fusion proteins, G-CSF-LE-Tf (LE) and G-CSF-$(H4)_2$-Tf (H4-2), and native G-CSF in BDF1 mice. PBS was used as vehicle controls. The doses of fusion proteins were adjusted to be molar equivalent to that of the native G-CSF. The ANC were determined at 24 h after administration. *Error bars* represent SEM, n = 4 for all groups.

1 mg/kg of G-CSF-equivalent (Fig. 4), which is consistent with our previous report (20). However, at these doses, subcutaneously injected G-CSF-$(H4)_2$-Tf exhibited a higher efficacy than both G-CSF and G-CSF-LE-Tf in absolute neutrophil count (ANC) (Fig. 4). At the low dose of 0.1 mg/kg of G-CSF-equivalent, the groups administered with both fusion proteins showed a higher ANC compared to the group receiving native G-CSF (Fig. 4).

In Vivo Characterization of the Fusion Proteins by Oral Administration

Native G-CSF (4 mg/kg), G-CSF-LE-Tf (20 mg/kg), and G-CSF-$(H4)_2$-Tf (20 mg/kg) were orally administered to each group of mice. These doses were lower than in our previous studies in which we used the dose of 10 mg/kg for native G-CSF (5 μmol/kg) and 50 mg/kg for G-CSF-LE-Tf (5 μmol/kg) (20). As shown in Fig. 5, at the relatively low dose in the current study, only the G-CSF-$(H4)_2$-Tf group showed a significant increase of ANC. Furthermore, the time course of

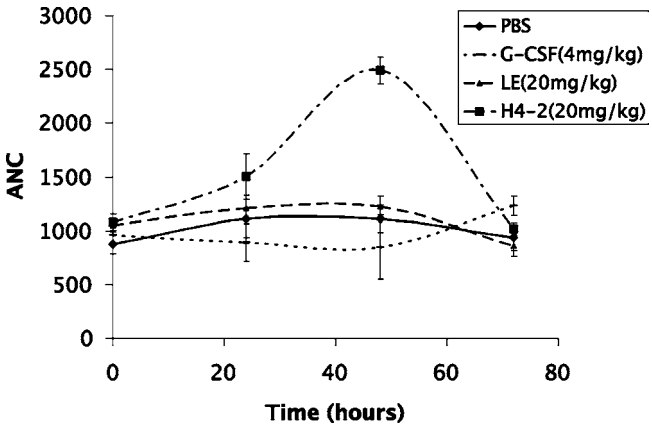

Fig. 5. Myelopoietic effect of orally administered fusion proteins, G-CSF-LE-Tf (LE) (20 mg/kg) and G-CSF-$(H4)_2$-Tf (H4-2) (20 mg/kg), as well as native G-CSF (4 mg/kg), in BDF1 mice. PBS was used as vehicle controls. The ANC were determined every 24 h. *Error bars* represent SEM, n = 4 for all groups.

response was different from that of the subcutaneous administration, with the peak of ANC appeared at 48 h (Fig. 5), instead of 24 h as seen in the subcutaneous injection (Fig. 4). Therefore, the fusion protein appearing to have a delayed myelopoietic effect when it was administered orally, which is consistent with our previous report (20).

DISCUSSION

The fusion protein, G-CSF-LE-Tf, which we used in our previous study, exhibited less than 10% of the *in vitro* activities of both TfR binding in Caco-2 cells and the proliferation in NFS-60 cells as compared to native Tf and G-CSF, respectively. Nevertheless, an effective myelopoietic activity of the fusion protein was detected when orally administered to BDF-1 mice (20). Since Tf and G-CSF in the fusion protein was linked by only a short LE dipeptide spacer, it is unlikely that these two protein domains can be separated in the body to release the fully active form of G-CSF. Therefore, the increase of ANC in BDF-1 mice most likely correlates directly to the *in vitro* cell proliferative activity of the fusion protein. Conceivably, if the *in vitro* activity of the fusion protein could be improved, the *in vivo* efficacy would be further enhanced.

There are two possibilities that the biological activity in the fusion protein will be reduced. First, it is possible that the two domains may sterically interfere with each other so that the accessibility of each domain to its respective receptor binding will be hindered. This steric hindrance could be avoided if a spacer peptide sequence would be inserted in the fusion protein to maintain a distance between the two functional domains (25). The second possibility is that the extended peptide sequence at either the amino- or the carboxyl-terminus of the functional domain in the fusion protein may decrease the affinity toward its receptor binding. In this case, the switch of the two domains from amino- and carboxyl-termini may preserve the conformation of the protein for the biological activity (31).

In order to increase the *in vitro* biological activity and, consequently, the *in vivo* myelopoietic effect of G-CSF-LE-Tf, we inserted different spacers to separate the Tf and G-CSF domains in the fusion protein. We chose five peptide sequences to construct the spacer between G-CSF and Tf domains, including peptides with random (GS), short α-helical (H2, H3, and H4), and long α-helical H4-2 conformation. From the NFS-60 proliferation results, all the fusion proteins with inserted spacer appeared to be more potent than G-CSF-LE-Tf (Fig. 3a). Among the spacers, the fusion protein with H4-2 spacer showed the highest cell proliferative activity in NFS-60 cell assay. It has been reported that spacers with helical conformation worked better than that with random conformation for maintaining the distance between functional domains in fusion proteins (24). In the case of G-CSF-$(H4)_2$-Tf, however, H4-2 not only provides a rigid, but also a longer spacer between G-CSF and Tf than in fusion proteins with H2, H3, and H4 spacers. Consequently, the EC_{50} of G-CSF-$(H4)_2$-Tf is about 10-fold lower than that of G-CSF-LE-Tf, even though it is still less effective than native G-CSF in *in vitro* proliferative assay (Fig. 3a).

On the other hand, there is no significant difference in TfR binding between G-CSF-LE-Tf and G-CSF-$(H4)_2$-Tf (Fig. 3b). The lack of improvement of TfR binding by inserting the spacer indicates that the decrease of TfR binding in the fusion protein is most likely due to the modification of the amino-terminus of Tf domain rather than a simple steric hindrance of the receptor binding site in the fusion protein. It has been demonstrated that binding of Tf to the target cell involves not only the receptor recognition but also the subsequent interaction of the N-terminal sequence of Tf with the membrane components (32). Therefore, the extra spacer peptide at the N-terminus may decrease the affinity of the membrane interaction and an extension of the spacer length may not be sufficient to increase the binding affinity of the fusion protein to the cell surface. Attempt has been made to reverse the sequence of G-CSF and Tf in the fusion protein, but the fusion protein produced from Tf-G-CSF expression in HEK293 cells was low in quantity and was inactive in NFS-60 cell proliferation assay (Unpublished results). The problem of low affinity of the fusion protein to TfR binding remains to be solved. It may pose as a limiting factor for further developing the Tf-fusion proteins into oral bioavailable protein drugs.

The *in vivo* myelopoietic effectiveness in both subcutaneous injection (Fig. 4) and oral administration (Fig. 5) correlated very well with the *in vitro* cell proliferative activity in cultured NFS-60 cells (Fig. 3a). In subcutaneous administration, the superiority of G-CSF-$(H4)_2$-Tf over native G-CSF in myelopoietic activity in BDF-1 mice is possibly due to a prolonged plasma half-life and a decrease of clearance as previously described in a recombinant G-CSF-albumin fusion protein (33). The advantage in pharmacokinetic parameters supersedes the fact that the fusion protein is less active than the native G-CSF in *in vitro* cell proliferation assay.

When the three forms of G-CSF and its Tf-fusion proteins were orally administered to BDF1 mice at a dose equivalent to 4 mg/kg of G-CSF, only the G-CSF-$(H4)_2$-Tf-treated mice group showed a significant increase of the ANC (Fig. 5). The dose in this study was significantly lower than that in our previous report. At this low dose, a delayed onset of the myelopoietic effect was observed in orally administered G-CSF-$(H4)_2$-Tf (Fig. 5), which is consistent with our previous report on oral administration of a higher dose of G-CSF-LE-Tf (20). It is known that the effect of G-CSF on ANC is not dose-dependent (34), and the effective curves in Fig. 4 clearly show the ceiling effect of G-CSF and the two fusion proteins. Therefore, it is difficult to estimate the bioavailability of orally administered G-CSF-$(H4)_2$-Tf by comparing effects on ANC in Figs. 4 and 5. A complete pharmacokinetic study must be performed in order to address the issue of bioavailability.

CONCLUSION

Different peptide spacers have been successfully inserted between the domains of G-CSF and Tf in production of recombinant fusion proteins. It was found that by inserting a long α-helix peptide, H4-2, a 10-fold decrease of the EC_{50} of *in vitro* cell proliferation response was observed, indicating an interference of the Tf domain with the G-CSF domain in the original fusion protein on the binding to its receptor. On the other hand, there was no improvement on the TfR binding activity of the fusion protein by the insertion of the spacers, indicating that the loss of TfR binding activity is not

due to a simple steric hindrance, but rather the alteration of the intrinsic conformation of the Tf domain in the fusion protein. However, the improvement of the *in vitro* G-CSF activity alone is sufficient to render an increase of the *in vivo* efficacy of the fusion protein in both subcutaneous and oral administration.

ACKNOWLEDGMENTS

We thank Dr. James Ihle, St. Jude's Children's Research Center, Memphis, TN, for the generous gift of the NFS-60 cell line and Dr. David K. Ann for his helpful discussion. This work was supported in part by NIH grants GM063647 and HL064365.

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