Recombinant granulocyte colony-stimulating factor–transferrin fusion protein as an oral myelopoietic agent

Yun Bai*, David K. Ann†, and Wei-Chiang Shen*‡

Departments of *Pharmaceutical Sciences and †Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, Los Angeles, CA 90033

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An expression construct harboring granulocyte colony-stimulating factor (G-CSF)–transferrin (Tf) fusion protein (G-CSF–Tf) was engineered by fusing human cDNAs encoding G-CSF and Tf to explore the feasibility of using Tf as a carrier moiety for oral delivery of therapeutic proteins. The recombinant protein, G-CSF–Tf, was harvested from protein-free, conditioned medium of transfected HEK293 cells. The *in vitro* studies demonstrated that the purified G-CSF–Tf fusion protein possesses the activity of both Tf receptor (TfR) binding in Caco-2 cells and G-CSF-dependent stimulation of NFS-60 cell proliferation. Subcutaneous administration of G-CSF–Tf fusion protein to BDF1 mice demonstrated a pharmacological effect comparable to the commercial G-CSF on the increase of absolute neutrophil counts (ANC). However, the fusion protein elicited a significant increase in ANC upon oral administration to BDF1 mice, whereas G-CSF had no effect. This study also showed that orally administered G-CSF–Tf elicits a sustained myelopoietic effect up to 3 days, whereas the s.c. administered G-CSF or G-CSF–Tf lasts only 1 day. Furthermore, coadministration of free Tf abolished the increase of ANC by orally delivered G-CSF–Tf, suggesting that the recombinant protein is absorbed via a TfR-mediated process in the gastrointestinal tract. Taken together, we conclude that the Tf-based recombinant fusion protein technology represents a promising approach for future development of orally effective peptide and protein drugs.

myelopoiesis | oral delivery | protein drug

Because of the progress of biotechnology, many recombinant peptides and proteins, such as human insulin (1), granulocyte colony-stimulating factor (G-CSF) (2), and erythropoietin (1, 2), are now available for clinical use with great efficacy. However, the administration of most protein drugs is limited to invasive methods, including i.v. or s.c. injection. Noninvasive delivery systems, especially for oral administration, of protein drugs have long been sought by the pharmaceutical industry, with little success (3, 4).

Transferrin (Tf), the natural transport protein for the delivery of iron to the cells, has been considered as a carrier in drug delivery for either crossing the blood–brain barrier or targeting to tumor cells (5–9). On the other hand, Tf receptor (TfR) is expressed abundantly in the human gastrointestinal (GI) epithelium (10), and Tf is relatively resistant to chymotryptic and tryptic digestion (11). Therefore, Tf has also been considered as a carrier for oral delivery of protein drugs (12, 13). Our previous studies unequivocally demonstrated that Tf-based chemical conjugation could be applied for noninvasive delivery of therapeutic proteins across the absorptive barriers, such as the small intestinal (14) and alveolar epithelial (12) cells, which express TfR on the surface. More importantly, a hypoglycemic effect was observed from using orally administered insulin-Tf conjugate in streptozotocin-induced diabetic rats (15, 16). Similarly, an increase of neutrophil number was observed when a Tf conjugate of G-CSF was administered orally to BDF1 mice (12, 13). However, the major obstacle with the current conjugation methodology is that the chemically cross-linked products are mostly heterogeneous mixtures of various size and composition (12) and, conceivably, are not suitable for therapeutic proteins.

In this report, the production of a functionally active G-CSF–Tf fusion protein by using the recombinant cDNA of human Tf and G-CSF is described. Oral administration in mice of the fusion protein prepared from a G-CSF–Tf-transfected HEK293 cells showed an effective GI absorption as demonstrated by the myelopoietic activity. The successful production of the G-CSF–Tf fusion protein as an orally active myelopoietic agent suggests the possibility of constructing various Tf fusion proteins to produce a series of therapeutic protein drugs with oral bioavailability.

Materials and Methods

Materials. Human Tf and other biochemicals were purchased from Sigma. G-CSF, as filgrastim, was a product of Amgen. The cell culture supplies and agents were obtained from Invitrogen.

Cell Lines. Murine myeloblastic NFS60 cells, kindly provided by James Ihle (St. Jude Children's Research Hospital, Memphis, TN), were grown in RPMI medium 1640 with 10% (vol/vol) FBS and 10% (vol/vol) WEHI-3 conditioned cell growth media. HEK293 cells, obtained from American Type Culture Collection, were grown as monolayers in MEM medium with 10% (vol/vol) FBS. Post G-CSF–Tf plasmid transfection of HEK cells, GIBCO TM CD293, a chemically defined protein-free medium, was used to simplify the downstream purification of the recombinant protein production. Caco-2 cells, obtained from American Type Culture Collection, were grown in DMEM supplemented with 10% (vol/vol) FBS. Human bladder carcinoma 5637 (American Type Culture Collection) was cultured in RPMI medium 1640 with 10% FBS.

Construction of G-CSF–Tf Plasmid. Human G-CSF cDNA, harboring the signal peptide was cloned by RT-PCR from human bladder carcinoma 5637 (American Type Culture Collection). 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.

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Abbreviations: ANC, absolute neutrophil count; G-CSF, granulocyte colony-stimulating factor; GI, gastrointestinal; Tf, transferrin; TfR, Tf receptor.

‡To whom correspondence should be addressed at: School of Pharmacy, University of Southern California, PSC 404B, 1985 Zonal Avenue, Los Angeles, CA 90033. E-mail: weishen@usc.edu.

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Engineering and Isolation of the Recombinant G-CSF–Tf Fusion Construct. For the production of the fusion protein, HEK293 cells were seeded in six-well cluster plates by using MEM supplemented with 10% FBS. After reaching 85–90% confluence, the cell monolayers were transiently transfected with G-CSF–Tf by using Lipofectamine according to the manufacturer's instructions (Invitrogen). The transfection mixture was changed to CD293 chemically defined protein-free medium after 4–6 h, and the cells were then cultured for 5 days, after which the medium was collected and the G-CSF–Tf fusion protein was isolated as the precipitate with 50% saturation of ammonium sulfate.

Western Blotting Analysis. Samples were separated by 8% SDS/PAGE. The proteins were transferred to a cellulose nitrate membrane (Millipore). Antibodies against human serum Tf (1:10,000) and human G-CSF (1:1,000) were used as primary antibodies. Horseradish peroxidase-conjugated anti-goat Ig antibody (1:10,000) was used as secondary antibody, and peroxidase activity was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia).

***In Vitro* Assay of G-CSF Proliferative Activity.** The G-CSF activity of the fusion protein was measured by NFS-60 proliferation assay (17, 18). NFS-60 cells were washed three times with RPMI medium 1640/10% FBS and aliquoted to 96-well microtiter plates at a density of 1×10^5 cells per ml. Subsequently, 10 μl of 10-fold serial dilutions of the G-CSF and fusion protein was added. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 h. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was subsequently performed essentially as described in ref. 19. Briefly, the cells were treated with 1 mg/ml MTT in serum-free and phenol red-free RPMI medium 1640 for 4 h. The formazan crystals that formed were then dissolved in isopropanol, and absorbance was measured at 570 nm on a TECAN GENios Plus microplate reader.

TfR Binding Assay. Human Tf was radiolabeled with ^{125}I (ICN), using chloramine-T catalyzed iodination, followed by purification using Sephadex G-50 column chromatography, and subsequently dialyzed in PBS (pH 7.8). Caco-2 cells were seeded in 12-well cluster plates until fully differentiated. Caco-2 monolayers were washed with cold PBS three times and then incubated in serum-free DMEM 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 unlabeled fusion protein or Tf in DMEM with 1 mg/ml BSA was added to different wells. After 30 min of incubation at 4°C, the medium was removed, and the cell monolayers were washed with cold PBS three times. The cells were then dissolved in 1 M NaOH, and the lysates were counted in a gamma counter.

***In Vivo* Studies.** Male BDF1 mice (Charles River Laboratories), 6–8 weeks of age, were used in all animal experiments described in this article. The mice were allowed to acclimate for 5 days. BDF1 mice were chosen for their stimulatory response to human G-CSF (17). Animal experiments were compliant with *Principles of Laboratory Animal Care* (National Institutes of Health Publication 85-23) and approved by the Institutional Animal Care and Utilization Committee of the University of Southern California. Before dosing, the mice were fasted for 12 h. The treatment groups (n = 3–4) received a single dose on day 0. The molecular mass of the fusion protein is approximately five times higher than G-CSF itself (G-CSF is 20 kDa, whereas Tf is 80 kDa); therefore, the final dosage for each had equal molar amounts. For s.c. administration, 5 mg/kg (0.05 μmol/kg) fusion protein or 1 mg/kg (0.05 μmol/kg) G-CSF was injected. For oral administration, 50 mg/kg (0.5 μmol/kg) fusion protein or 10 mg/kg (0.5 μmol/kg) G-CSF was given via a gavage needle. The volume for oral administration depended on the body weight of the mouse and ranged from 0.2 to 0.25 ml.

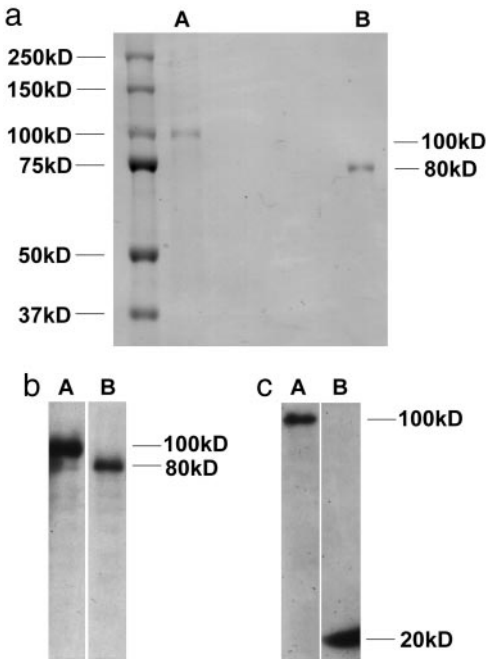

Fig. 1. Expression and identification of G-CSF–Tf fusion protein. (*a*) SDS/PAGE of purified recombinant fusion protein. Lane A, fusion protein; lane B, Tf. (*b*) Recognition of the recombinant fusion protein by anti-Tf and anti-G-CSF antibodies; Western blot using anti-Tf antibody. Lane A, fusion protein; lane B, Tf. (*c*) Western blot using anti-G-CSF antibody. Lane A, fusion protein; lane B, G-CSF control.

Blood samples were collected daily from the tail vein, diluted 20-fold, 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 neutrophils (PMN) among the leukocytes was determined manually by using Wright-stained blood smear glass slides that were examined under an Olympus BH-2 microscope. The absolute neutrophil count (ANC) was determined by multiplying the total WBC count by the PMN percentage (13).

Statistical Analysis. The statistical significance of the differences between experimental groups was determined by using the unpaired Student t test. Findings with two-tailed $P < 0.05$ were regarded as significant.

Results

Expression, Purification, and Biochemical Characterization of the Fusion Protein. After transfection, HEK293 cells were cultured in CD293 medium for 5 days, and the fusion protein was detected by performing PAGE analysis of the collected conditioned medium (Fig. 1*a*). One major band of ≈90% abundance, with a molecular mass of ≈100 kDa, was visualized by Coomassie blue staining after enrichment with 50% ammonium sulfate precipitation. The estimated molecular mass of the protein is consistent with that of the fusion protein, i.e., 80 kDa for Tf and 19.6 kDa for G-CSF.

The identity of the secreted fusion protein was confirmed by using both anti-Tf and anti-G-CSF antibodies in Western blot as illustrated in Fig. 1 *b* and *c*. Fig. 1*b* shows that the fusion protein (lane A) was recognized by anti-Tf antibody. Fig. 1*c* shows that the fusion protein (lane A) was also recognized by an anti-human G-CSF monoclonal antibody.

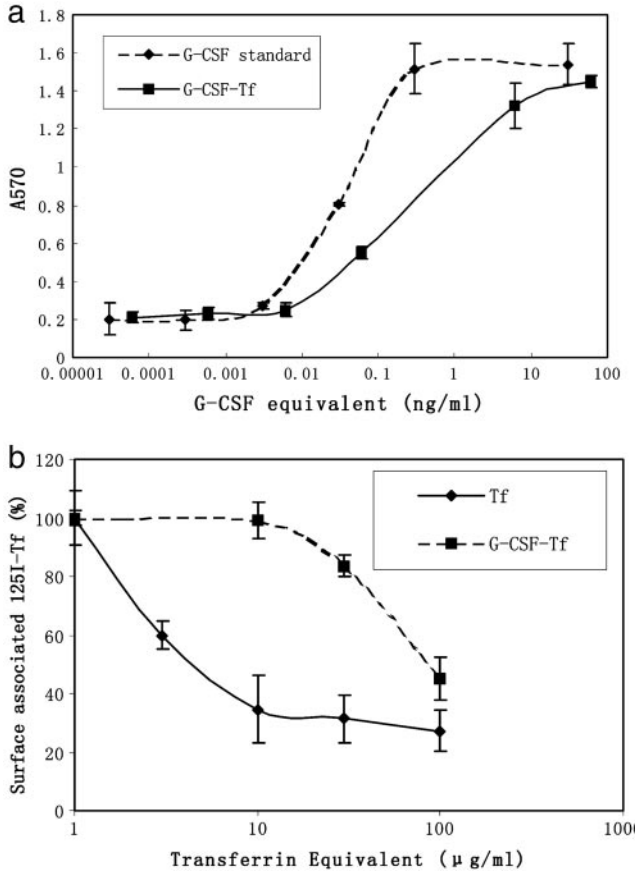

Fig. 2. *In vitro* study of G-CSF–Tf fusion protein activity. (*a*) Evaluation of G-CSF activity of the purified G-CSF–Tf fusion protein. Proliferation of the murine myeloblastic cell line NFS-60 was measured via MTT assay. The concentration of the fusion protein was expressed as the G-CSF equivalence. Error bars represent SD. (*b*) Evaluation of Tf activity of the purified G-CSF–Tf recombinant fusion protein. ^{125}I-labeled Tf (3 μg/ml in serum-free medium with 1 mg/ml BSA) was added to Caco-2 monolayers. Different concentrations of unlabeled fusion protein were added to compete for TfR binding. Error bars represent SD.

***In Vitro* G-CSF and Tf Activity of the Fusion Protein.** The biological activity of the purified fusion protein was assayed for G-CSF activity by determining its ability to stimulate NFS-60 proliferation. A different amount of fusion protein, which was sterile-filtered and normalized for G-CSF equivalency, was included in NFS-60 cell culture medium to replace G-CSF as a cell growth factor. As shown in Fig. 2*a*, the biological activity of the fusion protein was ≈1/10th of the commercial G-CSF, filgrastim. The EC_{50} of G-CSF control was ≈0.1 ng/ml, whereas the EC_{50} of the fusion protein was ≈1 ng/ml as a G-CSF equivalent.

The TfR binding ability of the G-CSF–Tf fusion protein was also determined. As shown in Fig. 2*b*, addition of unlabeled fusion protein caused a decrease in binding of ^{125}I-labeled Tf to TfR in cultured Caco-2 cells, indicating that the fusion protein maintained specific binding ability to TfR, even though the binding affinity was only ≈1/16th of that of Tf.

***In Vivo* Studies.** BDF1 mice were injected s.c. with 1 mg/kg G-CSF, 5 mg/kg G-CSF–Tf , or PBS control. The molecular mass of the fusion protein is approximately five times higher than G-CSF itself (G-CSF is 20 kDa, whereas Tf is 80 kDa); therefore, the final dosage for each is 5 μmol/kg. The day of dosage administration was denoted as day 0. As shown in Fig. 3, the fusion protein exhibited a comparable therapeutic effect to that

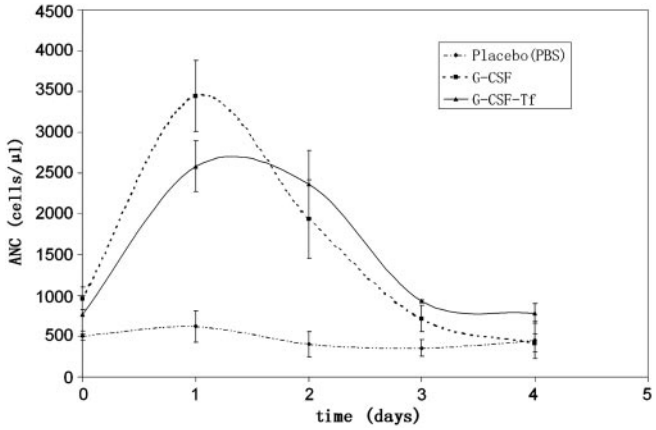

Fig. 3. Myelopoietic effect of s.c. administered fusion protein, G-CSF, or control. G-CSF (1 mg/kg) and fusion protein (5 mg/kg) were injected s.c. into BDF1 mice. Blood samples were tested to determine ANC daily. Error bar represents SEM (n = 3 for control and G-CSF; n = 4 for fusion protein). Student's t test (at day 1): placebo group vs. G-CSF group, P = 0.04; placebo group vs. fusion protein group, P = 0.004; G-CSF group vs. fusion protein group, P = 0.44.

of G-CSF. The time-effective curves of the fusion protein and G-CSF were similar; both G-CSF and G-CSF–Tf conferred maximum effect at day 1 ($P > 0.1$).

For the oral dosage experiments, BDF1 mice were given 10 mg/kg G-CSF, 50 mg/kg fusion protein, or the PBS vehicle via a gavage needle (day 0). As shown in Fig. 4, oral administration of G-CSF did not result in a statistically significant change ($P > 0.9$) in neutrophil level compared to the control (219 ± 85 cells per μl and 311 ± 97 cells per μl at day 1 for G-CSF treatment and control, respectively). However, mice that received the fusion protein demonstrated a significant elevation in ANC, 1,112 ± 232 cells per μl at day 1 (fusion protein vs. control, $P < 0.1$ at day 1). Furthermore, the ANC of the group treated with fusion protein increased to 1,643 ± 575 cells per μl 2 days after oral administration and only returned to the baseline as long as 5 days after administration.

To confirm that the *in vivo* transport of the fusion protein across the GI epithelia into the blood circulation was mediated

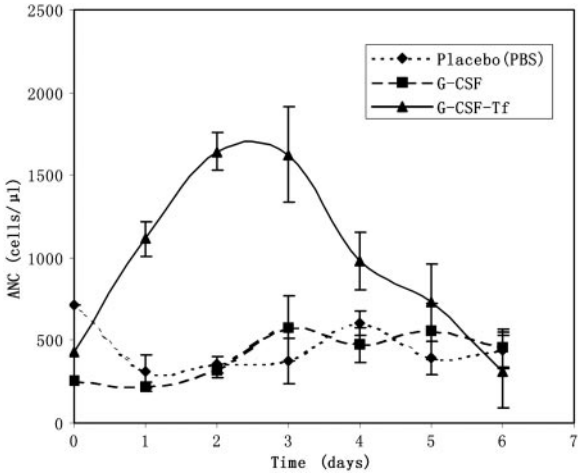

Fig. 4. Myelopoietic effect of orally administered fusion protein, G-CSF, or control. G-CSF (10 mg/kg) and fusion protein (50 mg/kg) were given orally via gavage needle to BDF1 mice. Blood samples were tested to determine ANC every 24 h. Error bar represents SEM (n = 3 for control and G-CSF; n = 4 for fusion protein). Student's t test (at day 2): placebo group vs. fusion protein group, P = 0.01; placebo group vs. G-CSF group, P = 0.9.

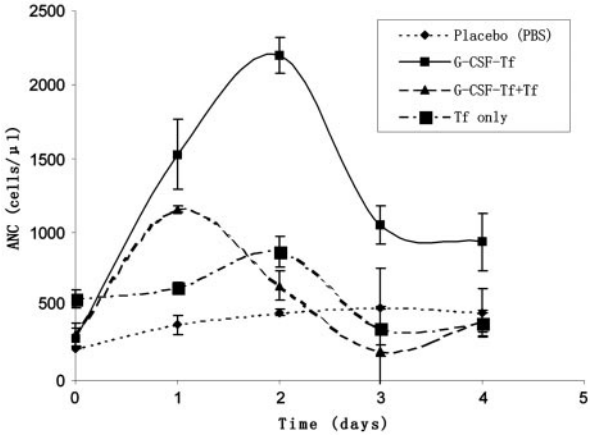

Fig. 5. Competition by free Tf on the absorption of orally administered G-CSF–Tf fusion protein. Fusion protein (50 mg/kg), fusion protein (50 mg/kg) plus Tf (500 mg/kg), Tf alone (500 mg/kg), and PBS as placebo were orally administered to BDF1 mice via a gavage needle. The myelopoietic effect was determined by daily ANCs. Error bar represents SEM ($n = 3$). Student's t test (at day 2): placebo group vs. fusion protein group, $P = 0.01$; placebo group vs. Tf competition group, $P = 0.1$; placebo group vs. Tf only group, $P = 0.3$.

via TfR, BDF1 mice were orally administered 50 mg/kg fusion protein in the presence or absence of a 10-fold excess of Tf (500 mg/kg). As shown in Fig. 5, a slight decrease of myelopoietic effect at day 1 was observed in the presence of a 10-fold excess of Tf. However, at day 2, the resultant ANC for fusion protein alone increased to 2,200 ± 120 cells per μl, whereas the ANC for fusion protein plus 10-fold excess of Tf had decreased to 642 ± 100 cells per μl, similar to the PBS control (on day 2, with Tf vs. without Tf: $P < 0.01$). Additionally, there was no significant difference between treatment with the G-CSF–Tf and G-CSF–Tf together with an excess of serum albumin (data not shown). Reduction of fusion protein absorption by Tf, but not by albumin, suggests that the fusion protein is absorbed in the GI tract via a TfR-mediated process.

Discussion

In this article, a recombinant fusion protein consisting of both human G-CSF and human Tf moieties was engineered and demonstrated to confer myelopoietic effect when orally delivered in an animal model. Recombinant fusion proteins have been reported to be effective in the development of oral vaccines (20, 21). However, this is the first demonstration, to our knowledge, that Tf moiety in Tf-based recombinant technology enhances the GI absorption of a therapeutic protein drug.

Even though the *in vitro* biological activity of the recombinant G-CSF–Tf approached only 1/10th of that by individual G-CSF (Fig. 2*a*) or Tf (Fig. 2*b*), the fusion protein exhibited a comparable myelopoietic effect to that of G-CSF when administered s.c. in mice. This elevated *in vivo* effectiveness may be due to a prolonged plasma half-life and a decrease of clearance as previously described in a recombinant G-CSF–albumin fusion protein (17). Conceivably, G-CSF–Tf may have a clearance rate similar to Tf, but not to G-CSF. This prolonged plasma half-life may compensate for the lower biological activity of the fusion protein. The fact that G-CSF–Tf exhibited a comparable *in vivo* pharmacological effect even though a much lower *in vitro* biological activity was observed suggests that a significant improvement of the therapeutic efficacy of G-CSF–Tf could be achieved if the biological activity of recombinant fusion protein could be augmented by other means. One possibility is to alter the length and/or amino acid composition of the linker between the G-CSF and Tf moieties. In the fusion protein described in this article, only a short linker of two amino acids (-Leu-Glu-) was inserted between the Tf and G-CSF moieties. Conceivably, such a short linker may decrease the chance for immunogenicity of the fusion protein; but it may also block the accessibility of the functional moiety for its receptor binding. It is noteworthy that a recombinant nerve growth factor–Tf fusion protein has been produced previously, in which a linker of IgG_3 hinge region was inserted between the two protein moieties to retain the *in vitro* biological activity (20).

The most striking finding in this study is that oral administration of the fusion protein elicited a clinically relevant increase in ANC, whereas oral G-CSF was totally ineffective (Fig. 4). The fusion protein, at an oral dose of 50 mg/kg, showed a 5-fold increase of ANC in BDF1 mice. Because G-CSF has been shown clinically to have a nonlinear dose–response (22, 23), a comparison of the area under the curve or bioavailability cannot be drawn here at this time. However, this study clearly demonstrates that the orally administered fusion protein does indeed have pharmacological effect. In addition, the orally administered fusion protein exhibited a prolonged effect on the neutrophil proliferation in mice. The increase of ANC for the oral administration of 50 mg/kg fusion protein in BDF1 mice was observed up to 4–5 days, whereas only up to 2 days for s.c. administration (Figs. 3 and 4). Interestingly, this sustained myelopoietic activity was not observed in the oral delivery of the G-CSF–Tf chemical conjugate (13). There are two possibilities that may account for the difference in the duration of the oral myelopoietic activity between the fusion protein and the chemical conjugates. First, the nonreducible characteristic of the fusion protein may lead to a longer half-life in serum, whereas free G-CSF may be released from the disulfide-linked conjugates after entering into the blood circulation (13, 24). Secondly, there may be a phenomenon such as a depot effect of the orally administered fusion protein in some organs, for instance, the liver or pancreas. These organs are targeted via the portal vein after GI absorption and are responsible for Tf storage, metabolism, and secretion (25). A depot effect could explain why the prolonged effect on myelopoiesis was not observed with s.c. administered fusion protein (Fig. 3). It is conceivable that the fusion protein entering the circulation from the injection site would have to compete with the high endogenous Tf concentration in the blood for receptor binding in various organs. Additionally, coadministration of a large excess of free Tf abolished the sustained myelopoietic effect of G-CSF–Tf and caused a shift of the ANC peak to day 1 (Fig. 5), a response that bears a resemblance to that of the s.c. injection (Fig. 3). The effect of free Tf on the myelopoietic effect of G-CSF–Tf suggests that the GI absorption of the fusion protein is a TfR-mediated process. However, the shortening of the duration of the myelopoietic effect may also suggest that a phenomenon such as a depot effect occurs in the liver or other organs because of the binding of the G-CSF–Tf to tissue-associated TfR. In this case, the excess Tf would complete with the fusion protein to the binding sites at the depot organ, although further work is needed to demonstrate such a competition between Tf and G-CSF–Tf.

In conclusion, results in this article demonstrate the feasibility that a recombinant G-CSF–Tf fusion protein can achieve both an oral bioavailability and an apparent sustained effect on myelopoiesis in BDF1 mice. These findings provide an approach for the future development of orally efficacious protein drugs. However, an elucidation of the exact mechanism of the absorption and the sustained duration effect is needed to expand and advance this approach to other protein drugs and, ultimately, to therapeutic applications.

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