The Transepithelial Transport of a G-CSF–Transferrin Conjugate in Caco-2 Cells and Its Myelopoietic Effect in BDF1 Mice

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Purpose. The purpose of this study was to investigate the transferrin-receptor (TfR)-mediated transepithelial transport of G-CSF–transferrin (Tf) conjugate in cultured enterocyte-like Caco-2 monolayers and the myelopoietic effect of subcutaneously and orally administered G-CSF–Tf in BDF1 mice.

Methods. Caco-2 monolayers exhibiting a minimum transepithelial electrical resistance of 500 $\Omega \cdot cm^2$ and BDF1 mice were used as *in vitro* and *in vivo* models, respectively. TfR-mediated transcytosis was measured by using ^{125}I-G-CSF–Tf and analyzing the downstream compartment by gamma counter. The efficacy of subcutaneously and orally administered G-CSF–Tf was determined by performing daily absolute neutrophil counts.

Results. Transport experiments in Caco-2 cells revealed that the monolayers that received ^{125}I-G-CSF–Tf exhibited significantly higher apical-to-basolateral transport rates compared to the monolayers that received ^{125}I-G-CSF. Inclusion of 100-fold excess unlabeled Tf reduced the extent of ^{125}I-G-CSF–Tf transport by 80%. Chromatographic and bioactivity assays revealed that the protein recovered from the basolateral compartment was the intact conjugate, and it retained full ability to stimulate the proliferation of the granulocyte-colony stimulating factor (G-CSF) dependent cell line, NFS-60, upon reduction. Subcutaneous administration of G-CSF–Tf in BDF1 mice demonstrated that the conjugate is able to elicit a statistically significant enhancement in therapeutic effect relative to filgrastim, which includes a longer duration of action with higher absolute neutrophil counts. Oral administration of G-CSF–Tf in BDF1 mice demonstrated that G-CSF–Tf is able to elicit a significant, and apparently dose-dependent, increase in absolute neutrophil counts whereas filgrastim had no effect.

Conclusions. Our data indicate that G-CSF–Tf is transported across Caco-2 monolayers by TfR-specific processes at a rate that is significantly higher than the nonspecific flux of G-CSF. G-CSF–Tf is also able to elicit a prolonged myelopoietic effect relative to filgrastim when administered subcutaneously or orally in BDF1 mice. The development of an orally bioavailable G-CSF has the potential to provide great benefit to patients under sustained G-CSF dosing regimes.

KEY WORDS: Caco-2; G-CSF-Tf; oral protein-drug delivery; transepithelial transport.

INTRODUCTION

Filgrastim (recombinant metHu G-CSF) is indicated in clinical conditions where it is desired to have increased production of circulating neutrophils. Common clinical indications include severe chronic neutropenia, bone marrow transplants, and patients undergoing chemotherapy for various cancers. One of the drawbacks of the current filgrastim therapy protocol is the need for daily or twice-daily injections. This comes as a result of the short half-life of the drug of only several hours (1).

Recently, several approaches have been taken that have sought to address the short half-life of filgrastim, including the pegylation of G-CSF (2) and the creation of fusion-protein constructs (3). Reduced clearance rates of the protein-drug have been reported for these modifications to G-CSF. However, these alterations to standard filgrastim dosing regimes are still limited to invasive administration.

The current subcutaneous filgrastim dosing regime is less than ideal for the patient because of the inconvenience and pain brought about by repeated injections. Noninvasive delivery methods have been a subject of interest for protein-based therapeutics as an alternative to the current subcutaneous or IV dosing. Nasal (4), rectal (5), pulmonary (6), ocular (7), and oral administration routes are among those that have been investigated. Among all of these, oral is the most preferred because it requires no specialized delivery device and is most convenient for the patient. However, oral delivery of protein-drugs is hampered by negligible bioavailability. Protein-based drugs are hampered by instability and proteolysis in the gastrointestinal tract. The large size and charged nature of the molecules also prevents them from traversing biological barriers. Coadministration and/or formulation with penetration enhancers and enzymatic inhibitors has been suggested as a means to achieve oral bioavailability of protein-drugs (8,9). However, the suitability of these methods for chronic usage remains questionable as they have been shown to be associated with adverse side-effects (10–12). As an alternative, natural transcytotic pathways, such as the transferrin receptor (TfR), have been used to enable the transepithelial delivery of large protein-based therapeutic molecules (13,14). Transcytosis of TfR does not alter cellular processes and would conceivably be less detrimental to membrane integrity than other methods that enable transport of protein-drugs. TfR is also a good candidate for targeting within the gastrointestinal tract as TfR has been reported to be highly expressed in the human GI epithelium (15). Transferrin (Tf) is also resistant to chymotryptic and tryptic digestion (16), two common proteolytic outcomes encountered by orally delivered protein-drugs. In addition, Tf is naturally released by the pancreas as part of normal digestive processes. This release of Tf by the pancreas is thought to facilitate the partial uptake of iron and regulation of iron homeostasis through the small intestine epithelium (13,17,18), thus providing a potential route of uptake for TfR-targeted protein-drug–Tf conjugates. Our laboratory has previously reported the hypoglycemic effect of an orally administered insulin–transferrin conjugate in diabetic mice (14). In this report, we describe the creation of a G-CSF–Tf protein conjugate that exhibits an increased duration of neutrophil proliferation action in mice (compared to filgrastim) with the added benefit of oral bioavailability.

MATERIALS AND METHODS

Caco-2 Cell Culture

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA; passage number 20) were grown on 24-mm-

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diamter, 0.4-μm pore-size polycarbonate Transwells (Costar, Cambridge, MA, USA) filters to confluence in Dulbecco's Modified Eagle Media supplemented with 10% FBS, glutamine, nonessential amino acids, penicillin, and streptomycin. All cell culture reagents were obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). The monolayers were allowed to differentiate for 7 days after reaching confluence. The transepithelial electrical resistance was measured with an epithelial voltohmmeter (EVOM, World Precision Instruments, West Haven, CT, USA). The monolayers typically established maximum resistances of 500 $\Omega \cdot cm^2$ after correcting for background membrane resistance.

Preparation of G-CSF-Tf Conjugate

In order to obtain an economical supply of G-CSF for the *in vivo* studies, human G-CSF was cloned by RT-PCR from human bladder carcinoma cell line 5637 (ATCC, Manassas, VA, USA) as described by Souza et al. (19) and subsequently sub-cloned into the pGEX-4T-1 expression vector (Amersham Pharmacia, Piscataway, NJ, USA) as a GST fusion protein in BL21 *Escherichia coli*. The expression of the fusion protein was accomplished by growing the transformed BL21 bacteria in LB broth at 37°C until A600 nm = 0.5 followed by induction of GST–G-CSF synthesis with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h. The bacteria were then harvested, resuspended in PBS, and lysed via sonication. Triton X-100 was added to the sonicate for a final concentration of 1% and gently mixed on ice for 30 min. GST–G-CSF fusion protein was purified from the crude sonicate with glutathione-Sepharose 4B, washed extensively with PBS, and incubated overnight with 5 U/mg of thrombin (Amersham) to liberate G-CSF from the matrix. The minute amount of thrombin was removed from the eluent via Benzamidine Sepharose 6B (Amersham) treatment. The purity of G-CSF was verified via SDS-PAGE, and the yield was estimated by measuring the absorbance at 280 nm, with an extinction coefficient of 15,820 M^{-1}. The yield of G-CSF from this procedure was typically 0.5 mg per liter of *E. coli* culture.

G-CSF was covalently linked to iron-loaded human Tf through disulfide linker chemistry as previously described (20). Briefly, a 20 mg/ml solution of iron-loaded Tf in PBS (pH 7.4) was mixed with a 10-fold molar excess of the heterobifunctional cross-linking agent *N*-succinimidyl 3-(2-pyriyldithio)propionate (SPDP) (Pierce, Rockford, IL, USA) at 4°C for 30 min. The final ratio of SPDP to Tf was determined to be 1:1. G-CSF was next modified with the homo-bifunctional linker agent 1,4-Di-[3′-(2'-pyridyldithio)propionamido]butane (DPDPB). The ratio of DPDPB to G-CSF was determined to be 1:1. SPDP-Tf was reduced with 25 mM dithiothreitol (Sigma, St. Louis, MO, USA) and reacted with 2-fold molar excess DPDPB–G-CSF to form the G-CSF–Tf conjugate. The reaction was quenched by addition of 1 mg/ml of *N*-ethylmaleimide, followed by overnight dialysis against PBS (pH 8.0, 4°C). The G-CSF–Tf conjugate was purified by gel filtration on a Sephacryl S-200 column. The molar ratio of G-CSF to Tf in the conjugate was determined by following the reaction spectrophotometrically at 343 nm, using 8.08×10^{-3} $M^{-1} \cdot cm^{-1}$ as the extinction coefficient for pyridine-2-thione and by SDS-PAGE densitometric scanning of the reduced conjugate (14).

Assessment of Apical-to-Basolateral Transcytosis of ^{125}I-Tf, ^{125}I-G-CSF, and ^{125}I-G-CSF–Tf Across Caco-2 Cell Monolayers

G-CSF was obtained for the *in vitro* transport studies by purifying r-metHu-G-CSF from commercial filgrastim preparations (Amgen, Thousand Oaks, CA, USA). Specifically, filgrastim solution was dialyzed against 10 mM acetate buffer, pH 4.0, to remove excipients. Following this, the recovered solution was concentrated in Amicon 3000 MWCO concentrators (Amicon, Bedford, MA, USA), and the G-CSF was iodinated by the chloramine-T catalyzed method. G-CSF and Tf–G-CSF were iodinated using the chloramine-T method (20). Transport studies were conducted on 2-week-old Caco-2 monolayers, 6 or 7 days after they had exhibited signs of tight junction development. Monolayers were washed once with DMEM and incubated at 37°C for 45 min to deplete endogenous Tf. Media were subsequently replaced and the monolayers were treated with ^{125}I-Tf, ^{125}I-G-CSF, or ^{125}I-Tf–G-CSF in the apical compartment (1.5 μg/ml). Nonspecific transport was measured in parallel by the inclusion of 100-fold molar excess of unlabeled Tf. At 2, 4, and 6 h postdosing, 500-μl samples were collected from the basolateral compartment and replenished with an equal volume of fresh DMEM. Samples were subjected to 15% trichloroacetic acid (TCA) precipitation, and radioactivity of the pellet was measured with a Packard gamma counter (Packard Instruments, Meriden, CT, USA). The extent of TfR-mediated transcytosis was determined by subtracting nonspecific transport (inclusive of excess unlabeled Tf) from total transport.

Analysis of Transcytosed Proteins

Transcytosed proteins were analyzed in separate transport studies via size exclusion chromatography and G-CSF–dependent cell proliferation assays. Basolateral fluids were collected after a 6-hour transport study as described above and subjected to Sephacryl S-200 column chromatographic analysis, where stock ^{125}I-labeled proteins (i.e.,^{125}I-Tf, ^{125}I-G-CSF, and ^{125}I-G-CSF–Tf) were used for determination of appropriate elution volumes. Biological activity assays were conducted for transcytosed ^{125}I-G-CSF and ^{125}I-G-CSF–Tf by measuring proliferation of the murine myeloblastic cell line NFS-60 (21). NFS-60 cells that had been cultured in RPMI-1640 medium, supplemented with both 10% FBS and 10% WEHI-3 (ATCC) conditioned medium (CM), were washed three times with serum- and WEHI-3 CM-free RPMI-1640 and aliquoted to 96-well microtiter plates at a density of 1×10^5 cells/ml. These cells were spiked with 20 μl of medium that had been previously recovered from the basolateral compartments in the conjugate transport studies and concentrated 10-fold with a Centricon centrifugal concentrator apparatus (Amicon, Bedford, MA, USA) and subjected to a brief reduction reaction (25 mM DTT, 15 min) to liberate free G-CSF. The samples were incubated at 37°C in a 5% CO_2 incubator for 48 h. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was subsequently performed essentially as previously described by Mosmann (22). Briefly, cells were incubated with phenol red–free RPMI media (Invitrogen) containing 1 mg/ml MTT for 2 h. The formazan crystals that formed were then dissolved in isopropanol (Sigma), and absorbance was measured at 570 nm on a Dynatech M70 microplate reader (Dynatech, Chantilly, VA, USA).

In vivo Studies

Specific pathogen-free BDF1 male mice (Charles River Laboratories, Wilmington, MA, USA) were used at 6–8 weeks of age. BDF1 mice were chosen due to their relatively robust stimulatory response to human G-CSF. The mice were allowed to acclimate for several days prior to beginning experiments. Animal experiments were compliant with the *Principles of Laboratory Animal Care* (NIH Publication No. 85-23) and approved by the IACUC at USC. The mice were fasted for 12 h prior to dosing. The mice were individually weighed, and the dosages were adjusted accordingly. The mouse weight was typically between 18–20 g. The treatment groups (n = 5) received a single subcutaneous (SC) injection or oral administration (via gavage needle) on day 0 with G-CSF–Tf, filgrastim, or vehicle control (PBS). Because the molecular weight of G-CSF–Tf is about 5-fold higher than that of filgrastim (filgrastim is 18.8 kDa whereas Tf is approximately 80 kDa), G-CSF–Tf was administered at 5 mg/kg SC, and filgrastim was given at 1 mg/kg SC. Similarly, G-SCF–Tf was given at a dose of 50 mg/kg orally and filgrastim was given at 10 mg/kg orally. The concentrations of the dosing solutions were adjusted so the dosage volume in all cases was 100 μl. G-CSF–Tf and filgrastim were formulated with sodium bicarbonate (30 mg/ml) in PBS prior to oral administration. Sodium bicarbonate was included to neutralize stomach acid in order to prevent hydrolysis of the protein-drug.

Blood samples were collected daily from the tails of the mice into microtubes that had been pretreated with heparin. Total white blood cell counts (WBC) were performed manually with a hemacytometer. The samples were diluted 20-fold and lysed in an acidic crystal-violet solution (0.1% crystal violet, 1% acetic acid, in water) prior to being loaded in the hemacytometer. The percentage of polymorphonuclear neutrophils (PMNs) among the leukocytes was determined manually with Wright-stained blood-smear glass slides that were examined with a 100× oil immersion lens on an Olympus BH-2 microscope (Olympus, Melville, NY, USA). The absolute number of neutrophils was determined by applying the observed PMN percentage against the total WBC count for each sample (23).

Statistical Analyses

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine statistical significance among group ($n \geq 3$) means using Tukey's post-hoc tests. $p < 0.05$ was considered significant.

RESULTS

Apical-to-Basolateral Transcytosis of G-CSF–Tf and TF Across Caco-2 Monolayers

Two-week-old Caco-2 monolayers, exhibiting transepithelial electrical resistance (TEER) levels of approximately 500 $\Omega \cdot cm^2$, were dosed with 1.5 μg/ml of ^{125}I-G-CSF–Tf or ^{125}I-G-CSF in the apical compartments of 6-well Transwells. As shown in Fig. 1, monolayers that received G-CSF–Tf exhibited significantly higher transport compared to the monolayers that received ^{125}I-G-CSF. For example, after 6 h, the amount of transported ^{125}I-G-CSF–Tf was 7.8-fold higher than ^{125}I-G-CSF (9.3 ± 0.8 fmol/well ^{125}I-G-CSF–Tf, 1.2 ± 0.7 fmol/well ^{125}I-G-CSF) (Fig. 1). In addition, the transport rate

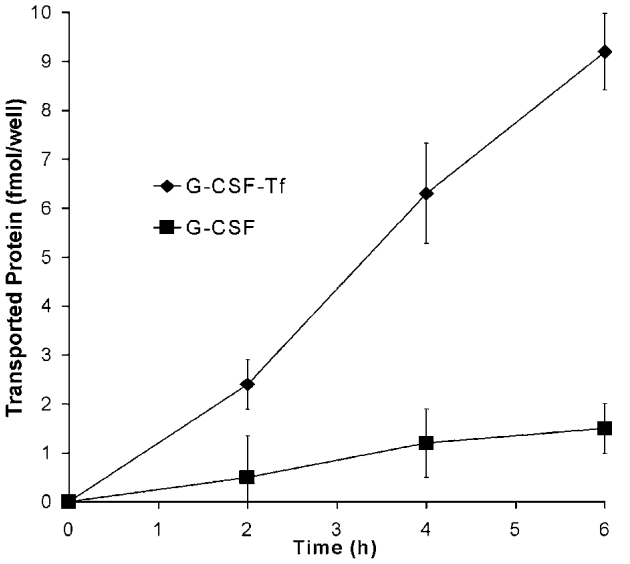

Fig. 1. Specific TfR-mediated transport of ^{125}I-GCSF-transferrin conjugate determined in Caco-2 monolayers and comparison to ^{125}I-GCSF. The apical compartments of 2-week-old Caco-2 monolayers were dosed with 1.5 μg/ml of ^{125}I-G-CSF–Tf or ^{125}I-G-CSF. Samples were taken from the basolateral compartments at regular intervals, subjected to 15% TCA precipitation, and radioactivity counted on a Packard gamma counter. Nonspecific ^{125}I-Tf transport was determined in parallel by the inclusion of 10-fold molar excess of nonradiolabeled Tf. The apical-to-basolateral transport of ^{125}I-GCSF was not affected by the presence of excess Tf whereas ^{125}I-G-CSF–Tf exhibited an 80% reduction in transport (data not shown). Error bars represent SEM (n = 3).

was also significantly higher, with ^{125}I-G-CSF–Tf transported at 1.7 fmol/well·h^{-1} and ^{125}I-G-CSF transported at 0.3 fmol/well·h^{-1}. Nonspecific ^{125}I-G-CSF–Tf transport was significantly lower than TfR-mediated transcytosis, with nonspecific transport composing 25% of the total transport for ^{125}I-G-CSF-Tf (data not shown).

Chromatographic Analysis of Transcytosed G-CSF–Tf

Apical compartments of Caco-2 monolayers were dosed with 1.5 μg/ml ^{125}I-G-CSF–Tf and the basolateral media collected after 6 h incubation at 37°C. Samples were subjected to size exclusion chromatography analysis. When samples recovered from the basolateral compartments were applied to a 40-ml Sephacryl-200 column, the major recorded peak coincided with the ^{125}I-G-CSF–Tf column standard at fraction 19, indicating that the molecular weight of the ^{125}I-G-CSF–Tf (recovered post-TfR-mediated transcytosis) was identical to the molecular weight of the ^{125}I-G-CSF–Tf standard (Fig. 2). The extent of degradation appeared to be minor, with relatively little small-molecule products appearing around fraction 40, accounting for 13% of total radioactivity.

Analysis of Biological Activity of Transcytosed ^{125}I-G-CSF–Tf in Caco-2 Monolayers

The biological activity of transcytosed conjugate was next determined through a NFS-60 MTT proliferation assay. Basolateral media was collected as described above after a 6-h transport experiment across Caco-2 monolayers. The samples were then sterile filtered, normalized for G-CSF content, and used as assay substrate. The sample was subjected to

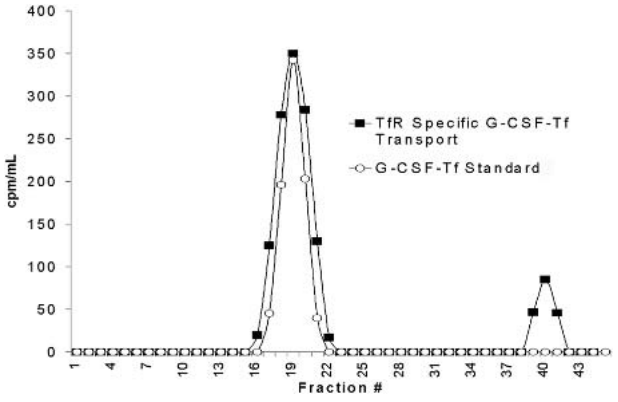

Fig. 2. Gel filtration chromatogram of TfR-mediated transcytosed ^{125}I-G-CSF–Tf. Apical compartments of Caco-2 monolayers were dosed with 1.5 μg/ml ^{125}I-G-CSF–Tf and the basolateral media collected after 6 h incubation at 37°C. The basolateral samples were applied to a 40 ml Sephacryl 200 column, eluted with PBS (pH 7.4), and compared to the standard ^{125}I-G-CSF–Tf solution. The fractions (1 ml) were subsequently counted on a Packard gamma counter.

reducing conditions prior to the assay (25 mM di-thio threitol, 15 min) in order to mimic reducing conditions that would encountered by the conjugate *in vivo.* The biological activity of the recovered ^{125}I-G-CSF–TF conjugate closely matches the activity of the G-CSF standard, with 0.1 ng/ml G-CSF equivalents displaying an absorbance (570 nm) of 0.87 ± 0.08 and 0.78 ± 0.06 for the G-CSF standard and the reduced ^{125}I-G-CSF–Tf conjugate, respectively (Fig. 3).

Neutrophil Proliferation in BDF1 Mice Dosed with Subcutaneous and Oral G-CSF–Tf

BDF1 mice were given 1 mg/kg filgrastim, 5 mg/kg G-CSF–Tf, or control vehicle subcutaneously. The day of dosage

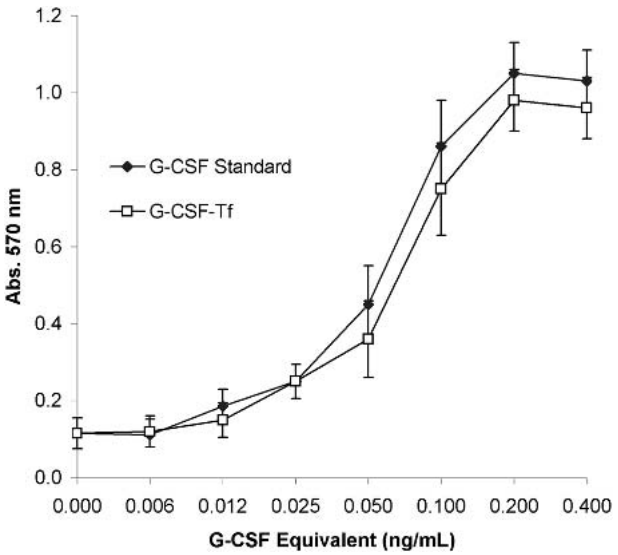

Fig. 3. Evaluation of biological activity of transcytosed G-CSF–Tf. Apical compartments of Caco-2 monolayers were dosed with 1.5 μg/ml ^{125}I-G-CSF or ^{125}I-G-CSF–Tf and the basolateral media collected after 6 h incubation at 37°C. Biological activity assays were conducted for transcytosed ^{125}I-GSCF–Tf after reduction of the disulfide linkage (25 mM DTT treatment of 15 min) by measuring proliferation of the murine myeloblastic cell line NFS-60 via MTT assay. Results are also shown for GCSF control (filgrastim). Error bars represent SEM (n = 3).

administration was denoted as day 0. By day 1, both the G-CSF–Tf and filgrastim treatment groups exhibited an increase in absolute neutrophil counts (2420 ± 450 cells/μl for the G-CSF–Tf treatment group and 2375 ± 400 cell/μl for the filgrastim treatment group) (Fig. 4). However, by day 2 there was a marked significant difference between the two treatment groups, with 4100 ± 510 cells/μl for the G-CSF–Tf treatment group and 1200 ± 385 cells/μl for the filgrastim treatment groups. The neutrophil levels remained elevated for the G-CSF–Tf treatment group, relative to control and filgrastim groups, through days 1 and 2 and then began to return to normal levels on day 3.

For the oral dosing experiments, BDF1 mice were given 10 mg/kg filgrastim, 50 mg/kg G-CSF–Tf, or control vehicle by gavage needle. The mice that received G-CSF–Tf demonstrated a statistically significant elevation in absolute neutrophil counts by day 1 with 2350 ± 225 cells/μl (Fig. 5). In contrast, oral administration of filgrastim did not result in a statistically significant change in neutrophil levels compared to control (1080 ± 280 cells/μl and 990 ± 95 cells/μl for filgrastim and control groups on day 1, respectively). Absolute neutrophil counts continued to be significantly elevated for the G-CSF–Tf treatment compared to control until day 3, when they returned to normal levels.

A dose-response experiment was next performed on orally administered G-CSF–Tf. BDF1 mice were given either 50 mg/kg, 25 mg/kg, or 12.5 mg/kg G-CSF–Tf orally by gavage needle. The group that received 50 mg/kg demonstrated a similar response as seen in the previous experiment, with 2300 ± 295 cells/μl recorded for absolute neutrophil counts on day 1 (Fig. 6), which was significantly different from the other two treatment groups. There was an indication that the neutrophil counts were elevated for the 25 mg/kg group compared to the 12.5 mg/kg group on day 1 (1400 ± 200 cells/μl and 1030 ± 168 cells/μl for 25 mg/kg and 12.5 mg/kg groups, respectively, $p \leq 0.10$).

DISCUSSION

Caco-2 cell culture model has come to serve as a leading qualitative indicator in screening GI permeability and absorp-

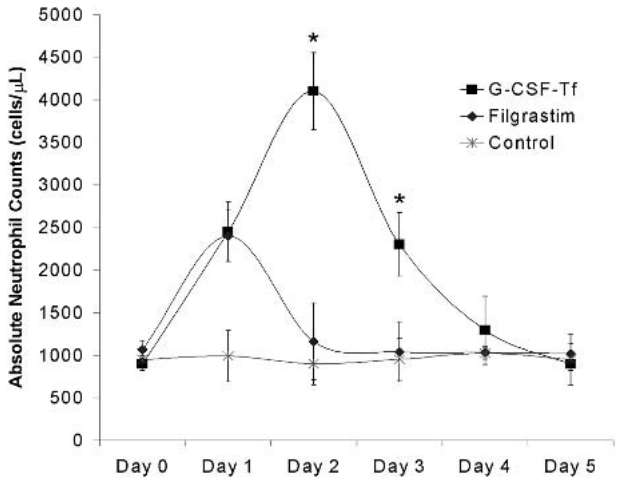

Fig. 4. Myelopoietic effect of subcutaneously administered G-CSF–Tf. G-CSF–Tf (5 mg/kg), filgrastim (1 mg/ml), or control vehicle was administered subcutaneously to 8-week-old male BDF1 mice. Absolute neutrophil counts were determined daily. * indicates $p < 0.01$. Error bars represent SEM (n = 5).

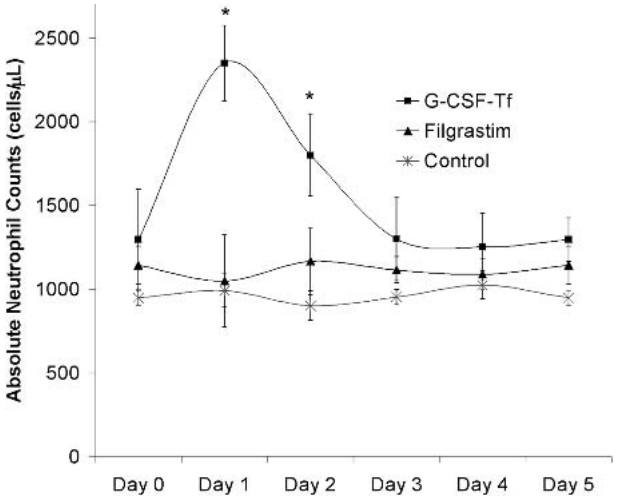

Fig. 5. Myelopoietic effect of orally administered G-CSF–Tf. G-CSF–Tf (50 mg/kg), filgrastim (10 mg/kg), or control vehicle was administered orally by gavage needle to 8-week-old male BDF1 mice. Absolute neutrophil counts were determined daily. * indicates $p < 0.01$. Error bars represent SEM ($n = 5$).

tion of therapeutic agents (24–27). In this report, we have investigated the amount of ^{125}I-G-CSF–Tf transported in the apical-to-basolateral direction, relative to ^{125}I-G-CSF, in 2-week-old Caco-2 monolayers (Fig. 1). Our data demonstrate that the rate of apical-to-basolateral ^{125}I-G-CSF–Tf transport is significantly higher than that of ^{125}I-G-CSF (1.70 fmol/well per h and 0.25 fmol/well per h for ^{125}I-G-CSF–Tf and ^{125}I-G-CSF, respectively). The enhanced transport of the G-CSF–Tf conjugate, compared to G-CSF, was presumed to be as a result of TfR-mediated transcytosis processes. This was suggested by competitive inhibition studies that demonstrated 80% reduction in ^{125}I-G-CSF–Tf apical-to-basolateral transport in the presence of 100-fold molar excess unlabeled Tf (data not shown). We have previously demonstrated that an insulin–Tf conjugate is able to be transcytosed across

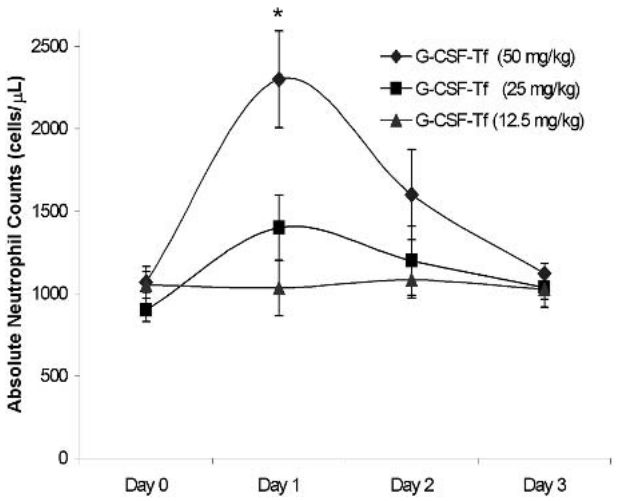

Fig. 6. Dose-response effect of orally administered G-CSF–Tf. G-CSF–Tf was given orally to 8-week-old BDF1 mice by gavage needle at doses of 50 mg/kg, 25 mg/kg, and 12.5 mg/kg. Myelopoietic effect was determined by daily absolute neutrophil counts. * indicates $p < 0.01$. Error bars represent SEM ($n = 5$).

Caco-2 monolayers by TfR-specific processes whereas the unconjugated insulin was unable to be transported across Caco-2 monolayers (28). The fact that we have demonstrated that G-CSF, a much larger protein-drug than insulin, is also able to transported across Caco-2 monolayers as a Tf-conjugate suggests that TfR-mediated transcytosis may serve as a multiplatform vehicle to deliver proteins of varying sizes.

Efforts were next undertaken to examine the protein-drug downstream after the transcytosis process. Our results indicate that G-CSF only suffered from a minor degree of degradation as a result of the TfR-mediated transcytosis process. For example, as shown in Fig. 2, when G-CSF–Tf is recovered from the basolateral compartment, post-TfR-mediated transport, and applied to a 40-ml S-200 column, the major recorded peak coincides with the peak for the G-CSF–Tf standard at fraction number 19. A low level of small-molecular-weight degradation products can also be seen at fraction number 40, accounting for only 13% of the total applied radioactivity. One can infer from this data that the molecular weight of the G-CSF–Tf conjugate is not altered by the transcytosis process in Caco-2 monolayers and relatively little conjugate is degraded. This result is supported by previous studies that have shown similar results in monolayers of various cell types and for different cargo-proteins (20,28,29).

In addition to determination of molecular weight, the biological activity of the transcytosed conjugate was also determined. The conjugate was recovered after a transcytosis experiment from the receiver compartment of Caco-2 monolayers, concentrated 10-fold, and then subjected to a brief mild DTT reduction (25 mM DTT, 15 min) in order to liberate free G-CSF from the conjugate. The DTT reduction also serves to mimic the natural reduction of disulfide-linked Tf protein–drug conjugates that one would observe *in vivo* (18). As shown in Fig. 3, the G-CSF that has undergone TfR-mediated transcytosis across Caco-2 monolayers retains almost all of its biological activity relative to control, as measured by the ability to stimulate the proliferation of NFS-60 cells (21) (ED_{50} values of 0.06 ng/ml and 0.07 ng/ml for the G-CSF standard and the transcytosed G-CSF, respectively). Taken together, these experiments suggest that TfR-mediated transport might be able to deliver protein-based therapeutics that are much larger than insulin (30) across GI epithelial barriers while retaining biological function of the cargo protein.

The *in vivo* efficacy of G-CSF–Tf was investigated next. Acute doses of G-CSF–Tf, filgrastim, or control vehicle were administered subcutaneously to 6–8-week-old BDF1 mice ($n = 5$). Absolute neutrophil counts were performed daily. As can be seen in Fig. 4, the G-CSF–Tf treatment group demonstrated an increased duration of action and significantly higher neutrophil counts (for days 2 and 3) relative to the group receiving filgrastim. G-CSF is known to follow a nonlinear pharmacokinetic clearance profile, which is most likely due in major part to receptor-mediated endocytosis (1). The clearance rate of G-CSF, when covalently conjugated to Tf, may be significantly reduced relative to filgrastim. This phenomenon could arise due to several factors. The first is that the conjugation of G-CSF to Tf may reduce or eliminate a predominant means of G-CSF clearance, which is dependent on the amount of circulating neutrophils (1). In addition, the large molecular weight of the conjugate, relative to G-CSF, should result in reduced renal clearance, which is the

other major route of G-CSF elimination. This hypothesis is supported by previous studies that have demonstrated a large decrease in clearance rate when G-CSF has been genetically fused to albumin (3) or when G-CSF has been modified by pegylation (2). In addition, the serum half-life of Tf in mice (40 h) (31) is much longer than the terminal half-life of G-CSF (~2.5 h) (32). It is possible that the G-CSF–Tf conjugate may have a clearance rate that is more similar to Tf than G-CSF, as Tf is predominantly recycled after binding to its receptor. G-CSF may also be slowly released from the conjugate as the disulfide linkage is reduced, as has been seen for other disulfide-linked protein-drug conjugates (18,33), enabling sustained neutrophilic leukocytosis relative to filgrastim. Another possibility is that subcutaneous administration of G-CSF–Tf may result in the binding of the conjugate to TfR in the interstitial tissues, creating a depot effect. G-CSF would also be slowly released from the site of administration in this situation, resulting in a sustained therapeutic effect.

In light of our findings that G-CSF–Tf can be specifically transported across Caco-2 monolayers (Figs. 1–3), the feasibility of using G-CSF–Tf in an oral route of administration was investigated. BDF1 mice (6–8 weeks old) were given G-CSF–Tf (50 mg/kg) or filgrastim (10 mg/kg) by gavage needle. Absolute neutrophil counts were performed as in the subcutaneous administration studies. Oral G-CSF–Tf was able to elicit a significant increase in neutrophil counts (for days 1 and 2) whereas filgrastim had no effect when administered orally (Fig. 5). In addition, G-CSF–Tf appears to have a dose-specific response when administered orally, providing evidence that G-CSF–Tf has a true pharmacological effect (Fig. 6). Because G-CSF will only have a myelopoietic effect if it is absorbed into the bloodstream, we presume from this data that G-CSF–Tf has a statistically significant oral bioavailability. In addition, when comparing the area under effect curve of subcutaneously and orally administered G-CSF–Tf (Figs. 4 and 5), the oral bioequivalence of G-CSF–Tf is about 4%, when judging on a therapeutic-effect vs. time basis without taking into account the rate of release of free G-CSF from the conjugate. As TfR is known to be highly expressed in the small intestine, and Tf is absorbed by the GI epithelium as a part of normal physiological processes (15,17), G-CSF–Tf may be taken up by similar processes. In addition, this result is supported by our previous report where we demonstrated that an orally administered insulin–Tf conjugate is able to elicit a hypoglycemic effect in diabetic rats whereas free insulin had no effect (18). G-CSF–Tf may therefore be able to be orally absorbed by TfR-specific transcellular transport processes.

In conclusion, we have described the creation of a G-CSF–Tf conjugate that is transported across Caco-2 monolayers by TfR-mediated transcytosis at a rate that is several-fold higher than nonspecific apical-to-basolateral G-CSF flux. G-CSF–Tf is also recovered from the receiver compartment of Caco-2 monolayers with the molecular weight intact and retains full biological function. The conjugate also exhibits a prolonged myelopoietic effect in mice compared to filgrastim. This result was observed in both subcutaneous and oral administration. The mechanism whereby G-CSF–Tf exhibits a prolonged therapeutic effect as well as the *in vivo* kinetics of G-CSF release from the conjugate remain to be determined. However, previous studies with PEG (2) and albumin (3) have indicated that conjugation to the relatively large-molecular-weight Tf may reduce the clearance rate of G-CSF. In addition to the improved pharmacokinetic characteristics of G-CSF–Tf, the development of an orally bioavailable G-CSF has the potential to provide great benefit for patients that are indicated for a chronic G-CSF dosing regime.

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