Transcytosis of GCSF-Transferrin across Rat Alveolar Epithelial Cell Monolayers

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Purpose. The purpose of this study was to use primary cultured rat alveolar epithelial cell monolayers to examine the potential of using transferrin receptor (TfR)-mediated transcytosis for noninvasive systemic protein drug delivery via the pulmonary route.

Methods. Freshly isolated rat type II pneumocytes were plated onto tissue culture-treated polycarbonate 12-mm Transwells. AEC monolayers ($\geq$ 2500 Ωcm^2) were treated with keratinocyte growth factor (10 ng/mL) for maintenance of type II cell-like characteristics. Filgrastim (GCSF)-Tf conjugates were prepared using the linkers SPDP and DPDPB. TfR-specific binding and uptake were determined using ^{125}I-Tf and ^{59}Fe-Tf treatment, respectively. Apical-to-basolateral (A-to-B) transferrin receptor (TfR)-mediated transcytosis was determined by dosing the apical compartment with 1.5 μg/mL of ^{125}I-Tf or ^{125}I-GCSF-Tf. Nonspecific TfR-independent transport of ^{125}I-Tf and ^{125}I-GCSF-Tf was determined in parallel by including 150 μg/mL of nonradiolabeled Tf. Basolateral samples (500 μL) were taken at 2, 4, and 6 h post-dosing, subjected to 15% trichloroacetic acid precipitation, and assayed in a Packard gamma counter. TfR-specific transport was determined as the difference between total and nonspecifc transport. The effects of brefeldin-A (BFA) on TfR distribution and (A-to-B) transport of ^{125}I-Tf, ^{125}I-GCSF and ^{125}I-GCSF-Tf was studied by including the agent in the apical fluid at 1 μg/mL.

Results. BFA treatment resulted in a small significant reduction in TfR at the basolateral surface of type II cell-like monolayers, while it had no effect on TfR distribution in type I cell-like monolayers. In contrast, BFA treatment significantly altered the endocytosis of TfR, reducing the basolateral uptake of ^{59}Fe-Tf while greatly increasing the apical uptake of ^{59}Fe-Tf. BFA treatment, however, did not affect the TfR-specific uptake of ^{59}Fe-Tf in type I cell-like monolayers. TfR-specific apical-to-basolateral transcytosis of ^{125}I-Tf and ^{126}I-GCSF-Tf conjugates was significantly enhanced in the presence of BFA in type II cell-like monolayers, whereas it had no effect on apical-to-basolateral transport of ^{125}I-GCSF. BFA-enhanced transport of ^{125}I-GCSF-Tf was approximately 3-fold higher than that of ^{125}I-GCSF in the presence or absence of BFA. Moreover, ^{125}I-GCSF transport in the presence of BFA was not significantly different from non-specific ^{125}I-GCSF-Tf transport. Chromatographic analyses and bio-assays revealed that GCSF-Tf was not degraded during transport via TfR-specific processes, and that GCSF retained biologic activity when liberated from the conjugate via dithiothreitol reduction.

Conclusion. This study suggests the possibility of using TfR-mediated transcytosis for systemic delivery of therapeutic proteins via the alveolar epithelium.

KEY WORDS: transferrin; alveolar epithelial cells; protein drug delivery; pulmonary drug delivery; BFA.

INTRODUCTION

The pulmonary epithelium has become an attractive target for systemic delivery of protein-based therapeutics. The large surface area (approx. 100 m^2) and extensive vascularization in the lung provide an ideal site for drug absorption. Several protein drugs have been reported to have relatively high bioavailabilities when delivered via the pulmonary route. However, conclusive knowledge regarding the mechanisms of transport through the pulmonary epithelial barrier remains elusive. In light of this, we have sought to develop a pulmonary delivery strategy that is targeted to a known transcytosis pathway, namely, transcytosis of the transferrin receptor (TfR), to obtain more predictable delivery qualities suitable for protein drugs of varying characteristics.

TfR-mediated drug delivery has advantages over other protein drug delivery strategies. For example, in contrast with penetration enhancers, TfR transcytosis neither alters the cellular plasma membrane nor disrupts the cellular tight junctions. As TfR endocytosis is an endogenous transport process, it is probable that a protein drug delivery scheme targeting TfR would have fewer side effects compared to other methods. In addition, transferrin is an advantageous protein carrier molecule because it is inherently resistant to enzymatic degradation processes (1–3). Primary cultures of rat type II alveolar epithelial cells (AECs) have been shown to exhibit many of the properties of alveolar epithelium *in vivo*, including apparent transdifferentiation from the type II cell-like to the type I cell-like phenotype within 3–4 days in culture (4–7). Type I pneumocytes comprise nearly 97% of the surface area of pulmonary alveoli, yet they are slightly outnumbered by type II cells. In addition to active Na^+ absorption afforded by both types of pneumocytes (8–10), the thin expansive type I cells are thought to primarily serve to enable efficient gas exchange, while the cuboidal type II cells exhibit essential secretory functions, such as secretion of pulmonary surfactant and surfactant proteins.

Primary cultured type II pneumocytes usually acquire the type I cell-like phenotype and morphology as the cells develop tight junctions leading to monolayers with high transepithelial electrical resistance (TEER) (4). This transition to the type I cell-like phenotype had retarded development of a model system for studying transport properties of monolayers with the type II cell (-like) phenotype. In this regard, keratinocyte growth factor (KGF) has recently been shown to cause retention of the type II cell-like phenotype in culture, while retaining high TEER levels (11–14). Thus, KGF-treated AEC monolayers were used in this study as a means to investigate the degree of TfR transcytosis in cells exhibiting the type II cell-like phenotype.

Our previous report (15) demonstrated that TfR expression is primarily limited to AEC monolayers of the type II cell-like phenotype, and that TfR expression is predominantly localized to the basolateral membrane. KGF treatment (10

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ng/mL) of AEC monolayers was found to restore TfR expression and TfR-mediated Tf endocytosis. Our current study utilizes these previous findings to develop a TfR-mediated protein drug delivery strategy that targets TfR in type II cell-like AEC monolayers.

MATERIALS AND METHODS

Primary Culture of Rat Alveolar Epithelial Cell Monolayers

Lungs of specific pathogen-free Sprague–Dawley male rats were perfused via the pulmonary artery and lavaged with Ca^{2+}/Mg^{2+}–free Ringer's solution. The lungs were then instilled with porcine pancreatic elastase (2.5 U/mL, Worthington Biochemical, Lakewood, NJ, USA) for 20 min at 37°C. Lung tissue blocks were minced to approx. 1 mm^3 size and sequentially filtered through 100-, 40-, and 10-μm Nitex membranes. Alveolar macrophages were removed from the crude cell mixture by IgG panning procedure (16). Partially purified type II pneumocytes (≥ 90% purity with ≥90% viability) were plated onto tissue culture-treated 12 mm polycarbonate filters (Transwell, 0.4-μm pore size, Costar Corning, Cambridge, MA, USA) at a density of 1.5 million cells/cm^2 in serum-free defined culture medium (MDSF) composed of a 1:1 mixture of DMEM and Ham's F-12 (Sigma Chemical, St. Louis, MO, USA) supplemented with 10% newborn bovine serum, 1.25 mg/mL bovine serum albumin, 100 U/mL penicillin, and 100 ng/mL streptomycin. Monolayers were fed on days 3 and 5 (or 6) with fresh culture medium. When KGF (hKGF, Calbiochem, San Diego, CA, USA) was added to the media bathing the AEC monolayers, it was present from day 0 onward at a concentration of 10 ng/mL and replenished with each feeding. TEER was measured with an epithelial voltage-ohm meter (EVOM, World Precision Instruments, Sarasota, FL, USA). Rat AEC monolayers typically achieved TEER ≥ 2,500 Ωcm^2 and PD ≥ 10 mV. Animal experiments were compliant with the 'Principles of Laboratory Animal Care' (NIH Publication #85-23) and approved by the IACUC at USC.

Measurement of Cell Surface TfR

Human Tf has been previously been reported to have high affinity for rat TfR and it was used as ligand to study TfR-specific processes (17,18). Human iron-loaded Tf (Sigma) was radiolabeled with ^{125}I (ICN, Irvine, CA, USA) using chloramine-T catalyzed modification (19), followed by purification by Sephadex G-50 column chromatography and subsequent dialysis in phosphate-buffered saline (PBS, pH 7.8). AEC monolayers were washed once with serum-free culture media (MDSF) supplemented with 0.1% bovine serum albumin and incubated at 37°C in the same media for 1 h to ensure removal of endogenous Tf. Monolayers were subsequently treated with brefeldin-A (BFA; 1.0 μg/mL) and incubated at 37°C for 2 h to allow for uptake of BFA. BFA-containing media were then removed and replaced with fresh MDSF containing 1.5 μg/mL ^{125}I-Tf in either the apical or basolateral compartment and incubated at 4°C for 2 h. Nonspecific binding was measured in parallel by the simultaneous addition of 100-fold excess unlabeled Tf in the respective bathing fluid. TfR-specific ^{125}I-Tf binding was determined as the difference between total and nonspecifically bound ^{125}I-Tf. After incubation, monolayers were washed three times with ice-cold PBS, pH 7.4. ^{125}I-Tf bound to the monolayers was measured with a Packard Gamma Counter (Packard Instruments, Meriden, CT, USA).

TfR-Dependent Uptake of ^{59}Fe

Human Apo-Tf (Sigma) was loaded with ^{59}Fe (Perkin–Elmer, Wellesley, MA, USA) as previously described (20). AEC monolayers were washed as in the ^{125}I-Tf binding studies described above. Monolayers were treated with BFA (1 μg/mL) in the apical compartment and subsequently dosed with 2 μg/mL ^{59}Fe-Tf on either the apical or basolateral surface. Non-TfR-mediated ^{59}Fe uptake was determined by parallel experiments that included 100-fold molar excess of unlabeled holo-transferrin in the apical fluid. AEC monolayers were incubated for 6 h with ^{59}Fe-Tf and then washed three times with ice-cold PBS. Cell monolayers were excised and associated ^{59}Fe was counted with a Packard Gamma Counter to determine uptake.

Preparation of Tf-GCSF Conjugate

GCSF (Amgen, Thousand Oaks, CA, USA) was covalently linked to iron-loaded human Tf through disulfide linker chemistry. 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 hetero-bifunctional cross-linking agent *N*-succinimidyl 3-(2-pyriyldithio)propionate (SPDP; Pierce, Rockford, IL, USA) at 4°C for 30 min. The reaction mixture was then dialyzed overnight against PBS (pH 8.0) to remove the excess SPDP. The final ratio of SPDP to Tf was determined to be 2:1. GCSF was next modified with the homo-bifunctional linker agent 1,4-Di-[3′-(2′-pyridyldithio)propionamido]butane (DPDPB; Pierce) by targeting the free sulfhydryl group of Cys17 in GCSF. A 10-fold molar excess of DPDPB was added to a 10 mg/mL solution of GCSF in PBS (pH 7.4) and allowed to react at room temperature until a maximum absorbance at 343nm was obtained. The DPDPB-modified GCSF was subsequently dialyzed overnight against PBS (pH 8.0, 4°C) to remove excess linker agent. The ratio of DPDPB to GCSF was determined to be 1:1. SPDP-Tf was reduced with 25 mM dithiothreitol (Sigma) to generate the reactive sulfhydryl species and reacted with 10-fold molar excess DPDPB-GCSF to form the GCSF-Tf conjugate with a 22.8 angstrom aliphatic spacer. Excess GCSF was needed to limit the extent of unwanted Tf-Tf crosslinking. 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 Tf-GCSF conjugate was purified by gel filtration on a Sephacryl S-200 column.

Assessment of Apical-to-Basolateral Transcytosis of ^{125}I-Tf, ^{125}I-GCSF and ^{125}I-Tf-GCSF across Rat Alveolar Epithelial Cell Monolayers

GCSF and Tf-GCSF were iodinated using the chloramine-T method as described above for the preparation of ^{125}I-Tf. Transport studies were conducted on day 6 or 7 monolayers, with or without KGF treatment from day 0. Monolayers were washed once with MDSF 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-GCSF or ^{125}I-Tf-GCSF in the apical compartment (1.5 μg/mL). Non-specific transport was measured in parallel by the inclusion of 100-fold molar excess of unlabeled Tf. BFA was added (1.0 μg/mL) to the apical compartment at the beginning of the transport study. At 2, 4, and 6 h post-dosing, 500 μL samples were collected from the basolateral compartment and replenished with an equal volume of fresh MDSF. Samples were subjected to 15% trichloroacetic acid precipitation and radioactivity of the pellet was measured with a Packard gamma counter. The extent of TfR-mediated transcytosis was determined by subtracting non-specific 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 GCSF-dependent cell proliferation assays. Basolateral fluids were collected after a 6-h 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-GCSF, and ^{125}I-GCSF-Tf) were used for determination of appropriate elution volumes. Biologic activity assays were conducted for transcytosed ^{125}I-GCSF and ^{125}I-GCSF-Tf by measuring proliferation of the murine myeloblastic cell line NFS-60 (21) (courtesy of Dr. J. Ihle, St. Jude's Children's Research Center, Memphis, TN, USA). 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). The samples were 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 subsequently performed essentially as previously described by Mosmann (22). Briefly, cells were incubated with phenol-red free RPMI media (Invitrogen, Carlsbad, CA, USA) containing 1 mg/mL MTT for 2 h. The formazan crystals that formed were then dissolved in isopropanol (Sigma) and absorbance was measured at 570nm on a Dynatech M70 microplate reader.

Statistical Analyses

Data are presented as mean (± SEM). One-way analysis of variance 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

Effect of BFA on AEC Monolayer Integrity

Treatment of AEC monolayers with BFA resulted in a decrease in transepithelial electrical resistance (TEER) to approximately 1 kΩcm^2 by the sixth hour. Although the TEER was significantly decreased upon BFA treatment, barrier function of the monolayers was maintained for the duration of the experiment (data not shown). BFA was thus further evaluated for its potential to be used as a TfR-dependent transcytosis enhancer in AEC monolayers of the type-II cell-like phenotype.

Effects of BFA on Distribution and Endocytosis of TfR

Day 6 KGF-treated type II cell-like monolayers and non-KGF-treated type-I cell-like monolayers were treated with BFA (1 μg/mL) for 2 h. The cell-surface distribution of TfR was subsequently detected by ^{125}I-Tf binding (1 μg/ml) at 4°C for 2 h. TfR-specific binding was determined by subtracting the non-specific component as measured by the separate inclusion of 100-fold excess non-radiolabeled Tf.

Non-KGF-treated AEC monolayers, or monolayers displaying the type I cell-like phenotype, did not exhibit significant differences in TfR expression at the apical or basolateral membranes, compared to control AEC monolayers, in response to BFA treatment (Fig. 1A). TfR-specific ^{125}I-Tf binding at the basolateral surface was 1.3 ± 0.3 and 1.5 ± 0.3 fmol TfR/cm^2, with and without BFA treatment, respectively. TfR-specific ^{125}I-Tf binding at the apical surface was 0.20 ± 1.9 and 0.19 ± 2.0 fmol TfR/cm^2, with and without BFA treatment respectively.

As shown in Fig. 1B, KGF-treated type II cell-like monolayers exhibited markedly increased TfR, although the inclusion of BFA (1 μg/mL) only slightly altered the static distribution of TfR in KGF-treated monolayers. Specifically, KGF-treated monolayers showed a small significant reduction in TfR at the basolateral membrane upon BFA treatment (10.5 ± 0.5 fmol TfR/cm^2) as compared with KGF-treated monolayers that did not receive BFA (11.8 ± 0.4 fmol TfR/cm^2). The apical membrane of KGF-treated AEC monolayers did not show significant differences in TfR expression upon BFA treatment, with BFA treated monolayers exhibiting 1.2 ± 2.0 fmol TfR/cm^2 and control monolayers exhibiting 1.74 ± 1.9 fmol TfR/cm^2.

Because ^{125}I-Tf binding at 4°C presents only a static view of receptor distribution, efforts were next undertaken to examine what effect BFA might have on endocytosis of TfR at the apical and basolateral membranes of type I and type II cell-like monolayers. ^{59}Fe-loaded Tf was applied to either the apical or basolateral compartment of day 6 monolayers (± KGF), with or without BFA (1.0 μg/mL), and incubated at 37°C for 5 h. The use of ^{59}Fe-Tf enabled endocytosis events to be measured, unlike the use of ^{125}I-Tf, since upon endocytosis of the ^{59}Fe-Tf-TfR complex, ^{59}Fe is known to disassociate from the complex in the acidic endosome and accumulate inside the cell (23). BFA had no effect on ^{59}Fe-Tf uptake in non-KGF-treated monolayers (Fig. 1C). However, BFA significantly altered the extent of endocytosis in KGF-treated monolayers at the apical and basolateral membranes as shown in Fig. 1D. For example, upon BFA treatment, ^{59}Fe accumulation at the basolateral membrane decreased from 225 ± 25 fmol ^{59}Fe/cm^2 to 175 ± 23 fmol ^{59}Fe/cm^2. In contrast, ^{59}Fe accumulation at the apical membrane increased upon BFA treatment to 70 ± 10 fmol ^{59}Fe/cm^2 as compared to a negligible uptake of 5.1 ± 8.2 fmol ^{59}Fe/cm^2 without BFA treatment.

TfR-Mediated Apical-to-Basolateral Tf Transport in BFA-Treated Rat Alveolar Cells

Transport studies were performed to determine if BFA treatment was able to elicit TfR-specific apical-to basolateral

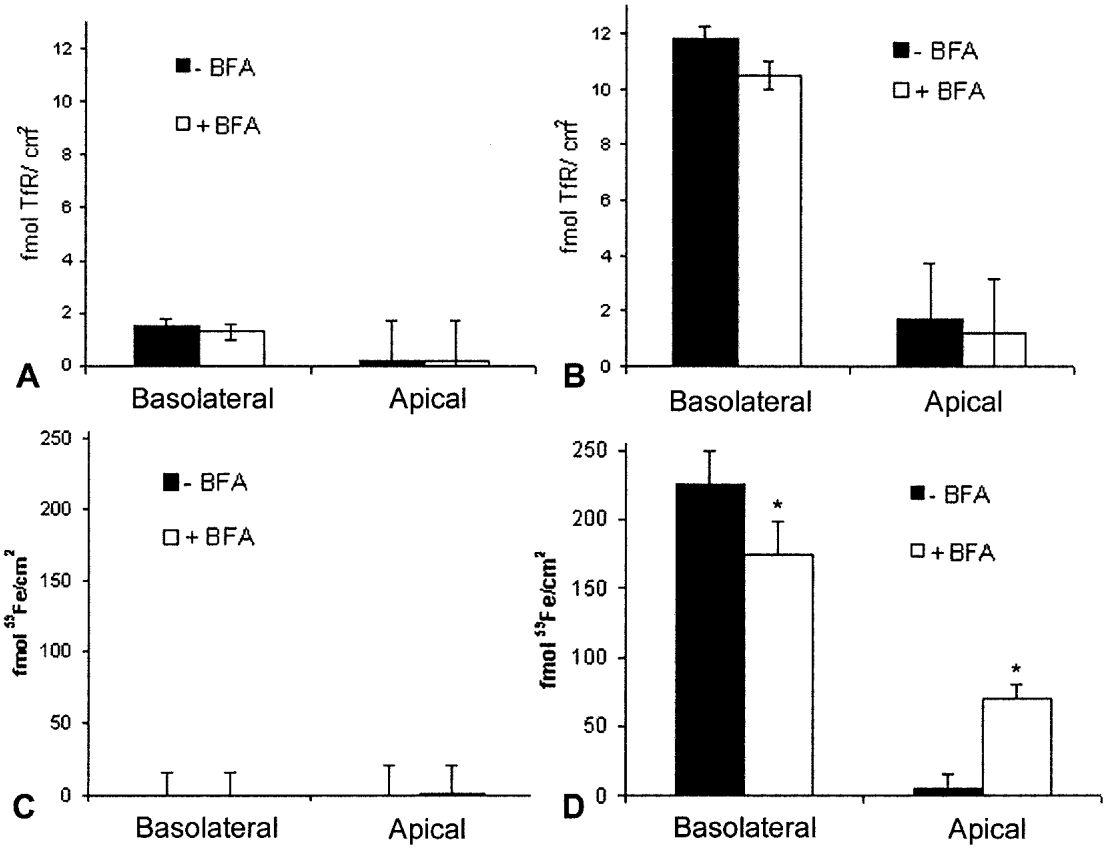

Fig. 1. The effects of brefeldin-A (BFA) on surface transferrin receptor (TfR) expression and ^{59}Fe-Tf uptake in rat alveolar epithelial cells (AECs). TfR levels were determined through TfR-specific binding of ^{125}I-Tf (1.5 μg/mL) to the apical and basolateral surfaces of AEC monolayers grown on 12-mm Transwell filters in the presence **(A)** or absence **(B)** of keratinocyte growth factor (10 ng/mL). Nonspecific binding was determined in parallel by the addition of 100-fold molar excess of nonradiolabeled Tf to the respective donor fluid. The surface binding experiments were conducted at 4°C for 2 h. TfR-specific binding was determined as the difference between total and nonspecific ^{125}I-Tf binding. The effect of BFA on TfR-mediated uptake was determined via ^{59}Fe-Tf endocytosis. ^{59}Fe-Tf (2 μg/mL) was added to either the apical or basolateral compartment of day 6 AEC monlayers in the presence **(C)** or absence **(D)** of keratinocyte growth factor (10 ng/mL). Nonspecific uptake was determined in parallel by the addition of 100-fold molar excess of non-radiolabeled Tf to the respective donor fluid. The monolayers were excised after a 6-h incubation and the level of radioactivity was determined on a Packard gamma counter. BFA was added to the apical compartment in all cases (1.0 μg/mL). TfR-specific uptake was determined as the difference between total and nonspecific ^{59}Fe uptake. In all cases, error bars represent SEM for $n = 6$. * indicates $p < 0.05$ compared with respective non-BFA-treated membrane surfaces as determined by one-way analysis of variance.

transcytosis in type II cell-like AEC monolayers (i.e., KGF-treated) and type I cell-like AEC monolayers (i.e., treated with control vehicle). Day 7 AEC monolayers (with or without KGF treatment) were dosed with 1.5 μg/mL of ^{125}I-Tf in the apical compartment. The apical compartments of the monolayers were subsequently spiked with BFA and incubated at 37°C. Samples were taken from the basolateral compartments at regular intervals, subjected to 15% trichloroacetic acid precipitation, and radioactivity was counted on a Packard gamma counter. Nonspecific ^{125}I-Tf transport was determined in parallel by inclusion of 150 μg/mL of unlabeled Tf. As shown in Fig. 2, KGF-treated monolayers demonstrated a significant increase in TfR-specific transcytosis in response to BFA treatment. For example, after 6 h, the amount of transported ^{125}I-Tf was 4.4-fold higher in the presence of BFA (0.31 ± 0.3 ng/well ^{125}I-Tf with BFA, 0.07 ± 0.02 ng/well ^{125}I-Tf without BFA; Fig. 2A). BFA treatment did not result in significant change in nonspecific transport (0.06 ± 0.3 ng/well ^{125}I-Tf with BFA, 0.05 ± 0.02 ng/well ^{125}I-Tf without BFA; data not shown). In contrast, the type I cell-like AEC monolayers (no KGF) did not demonstrate significant TfR-mediated ^{125}I-Tf transcytosis in the absence or presence of BFA (Fig. 2B).

TfR-Mediated ^{125}I-GCSF-Tf Transcytosis in Type II Cell-Like AEC Monolayers

^{125}I-GCSF-Tf transport studies were performed KGF-treated day 7 AEC monolayers. The transport studies were performed in an analogous manner to the ^{125}I-Tf studies, with the apical compartment dosed with 1.5 μg/mL ^{125}I-GCSF-Tf and then spiked with BFA (1.0 μg/mL). As shown in Fig. 3A, BFA significantly enhanced TfR-specific apical-to-basolateral transcytosis of ^{125}I-GCSF-Tf (3.7 ± 0.68 fmol/well ^{125}I-GCSF-Tf after 6 h), whereas those monolayers that were not exposed to BFA displayed negligible TfR-specific ^{125}I-GCSF-Tf

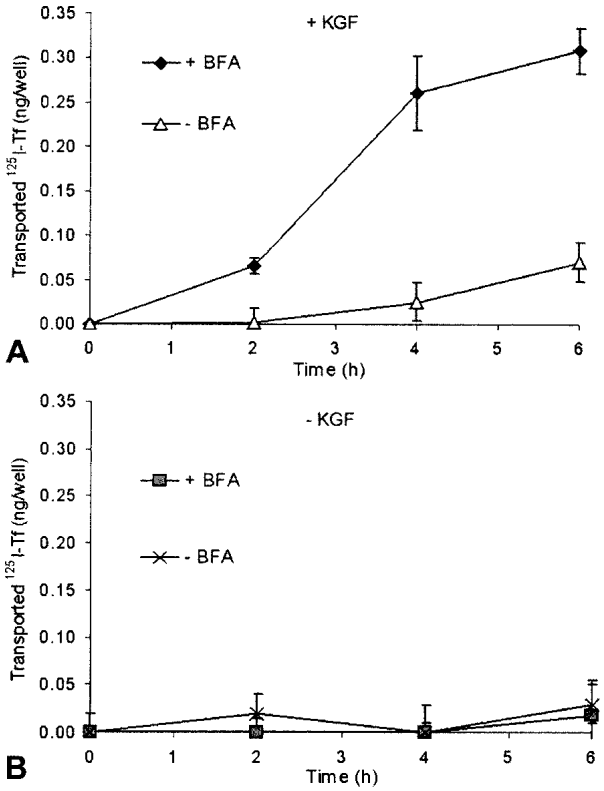

Fig. 2. The effects of brefeldin-A (BFA) on ^{125}I-Tf transport. Day 7 alveolar epithelial cell monolayers with **(A)** or without **(B)** keratinocyte growth factor (KGF) treatment were dosed with 1.5 μg/mL of ^{125}I-Tf in the apical compartment. The apical compartments of the monolayers were subsequently spiked with BFA and incubated at 37°C. Samples were taken from the basolateral compartments at regular intervals, subjected to 15% trichloroacetic acid (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 non-radiolabeled Tf. BFA (1.0 μg/mL) significantly enhanced transferrin receptor-mediated apical-to-basal transport in KGF-treated (10 ng/mL) alveolar epithelial cell monolayers, yet had no effect on monolayers not treated with KGF. KGF-treated cells exhibited a small amount of transport in the absence of BFA. BFA treatment did not result in significant change in nonspecific transport (0.06 ± 0.3 ng/well ^{125}I-Tf with BFA, 0.05 ± 0.02 ng/well ^{125}I-Tf without BFA; data not shown). n = 6.

transcytosis (0.21 ± 0.45 fmol/well ^{125}I-GCSF-Tf after 6 h). BFA treatment did not significantly affect the degree of nonspecific ^{125}I-GCSF-Tf transport (0.67 ± 0.3 fmol/well ^{125}I-GCSF-Tf with BFA, 0.61 ± 0.3 ng/well ^{125}I-GCSF-Tf without BFA; data not shown).

Comparison of ^{125}I-GCSF-Tf and ^{125}I-GCSF Apical-to-Basolateral Transport in AEC Monolayers

The efficacy of BFA-induced enhancement of ^{125}I-GCSF-Tf apical-to-basolateral transcytosis was compared to the apical-to-basolateral transport of ^{125}I-GCSF. Transport studies were performed as described above, with the apical compartments of day 7 type II cell-like monolayers dosed with 1.5 μg/mL of ^{125}I-GCSF and spiked with BFA (1.0 μg/mL) at the beginning of the transport study. TfR-specific transport of ^{125}I-GCSF-Tf (+ BFA; Fig. 3A) was significantly greater than that of the similarly treated ^{125}I-GCSF group (+

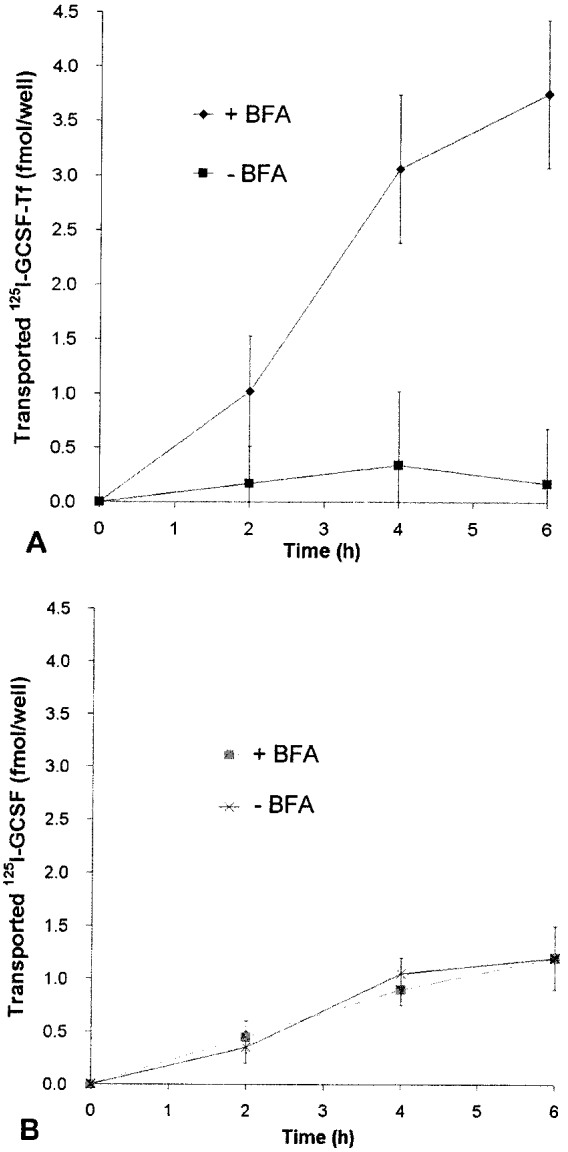

Fig. 3. Specific TfR-mediated transport of ^{125}I-GCSF-transferrin conjugate determined on day 7 and comparison with ^{125}I-GCSF. The apical compartments of day 7 type II cell-like monolayers were dosed with 1.5 μg/mL of ^{125}I-GCSF-Tf **(A)** or ^{125}I-GCSF **(B)** and spiked with brefeldin-A (BFA; 1.0 μg/mL) at the beginning of the transport study. 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. BFA (1.0 μg/mL) significantly enhanced TfR-mediated apical-to-basolateral transport in keratinocyte growth factor -treated (10 ng/mL) alveolar epithelial cell monolayers. Monolayers that were not exposed to BFA displayed negligible transferrin receptor-mediated transcytosis. BFA treatment did not significantly affect nonspecific ^{125}I-GCSF-Tf transport (0.67 ± 0.3 fmol/well ^{125}I-GCSF-Tf with BFA, 0.61 ± 0.3 ng/well ^{125}I-GCSF-Tf without BFA; data not shown). The apical-to-basolateral transport of ^{125}I-GCSF was not affected by the presence of BFA, exhibiting nearly identical transport profiles with or without BFA. n = 6.

BFA; Fig. 3B), with respective cumulative 6-h apical-to-basolateral transports of 3.7 ± 0.68 fmol/well ^{125}I-GCSF-Tf and 1.2 ± 0.11 fmol/well ^{125}I-GCSF. The apical-to-basolateral transport of ^{125}I-GCSF was not affected by the presence of

BFA, exhibiting nearly identical transport profiles with or without BFA.

Analysis of Transcytosed ^{125}I-GCSF-Tf

Apical compartments of BFA-treated (1.0 μg/mL) type II cell-like AEC monolayers were dosed with 1.5 μg/mL ^{125}I-GCSF-Tf and the basolateral media collected after 6-h incubation at 37°C. Samples were subjected to size exclusion chromatography analysis and assayed for bioactivity by measuring the ability to stimulate NFS-60 cell proliferation (21).

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-GCSF-Tf column standard at fraction 19, indicating that the molecular weight of the ^{125}I-GCSF-Tf (recovered post-TfR-mediated transcytosis) was identical to the molecular weight of the ^{125}I-GCSF-Tf standard (Fig. 4). The extent of degradation appeared to be minor, with relatively little small-molecule products appearing around fraction 40, accounting for 14% of total radioactivity.

The biologic activity of transcytosed conjugate was next determined through a NFS-60 MTT proliferation assay. Basolateral media was collected as described above. The samples were then sterile filtered, normalized for GCSF content, and used as assay substrate. The recovered conjugate displayed relatively minor proliferative ability, with 0.1 ng/mL GCSF equivalents displaying an absorbance (570 nm) of only 0.23 ± 0.5 (Fig. 5). However, when the sample is subjected to reducing conditions prior to the assay (25 mM DTT, 15 min), we find that biologic activity is recovered and fairly closely matches activity of the GCSF standard, with 0.1 ng/mL GCSF equivalents displaying an absorbance (570 nm) of 0.95 ± 0.07 and 0.80 ± 0.05 for the GCSF standard and the reduced ^{125}I-GCSF-Tf conjugate, respectively.

DISCUSSION

We previously reported that Tf-protein drug conjugates have been successfully used to facilitate the transcytosis of the protein drugs across epithelial barriers *in vitro* and *in vivo*

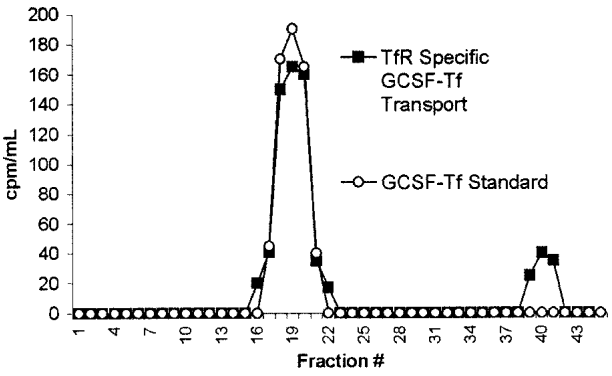

Fig. 4. Gel filtration chromatogram of transferrin receptor-mediated transcytosed ^{125}I-GCSF-Tf. Apical compartments of BFA-treated (1.0 μg/mL) type II cell-like AEC monolayers were dosed with 1.5 μg/mL ^{125}I-GCSF-Tf and the basolateral media collected after six hour incubation at 37°C. The basolateral samples were applied to a 40 mL Sephacryl 200 column, eluted with PBS (pH 7.4), and compared to the standard ^{125}I-GCSF-Tf solution. The fractions (1 mL) were subsequently counted on a Packard gamma counter.

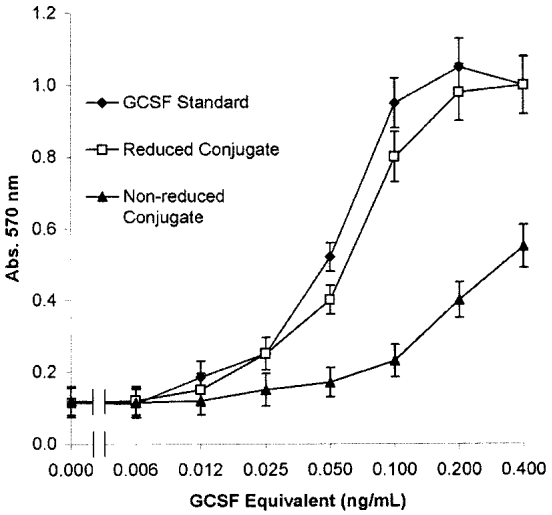

Fig. 5. Evaluation of biologic activity of transcytosed GCSF-transferrin. Apical compartments of brefeldin-A-treated (1.0 μg/mL) type II cell-like alveolar epithelial cell monolayers were dosed with 1.5 μg/mL ^{125}I-GCSF or ^{125}I-GCSF-Tf and the basolateral media collected after 6-h incubation at 37°C. Biologic activity assays were conducted for transcytosed ^{125}I-GCSF-Tf or reduced ^{125}I-GSCF-Tf (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 (Neupogen).

(24,25). Although past efforts focused on the development of oral protein drug delivery methods, the current study explores the possibility of noninvasive protein drug delivery facilitated by transferrin-receptor transcytosis across the pulmonary epithelium. One of the limitations of using Tf as a carrier molecule for pulmonary delivery of protein-based therapeutics is the predominant localization of TfR to the basolateral membrane of alveolar epithelium (15). There is very little transcytosis of TfR from the basolateral membrane to the apical membrane under normal conditions. Indeed, epithelial tissues will in general have TfR predominantly localized to the basolateral (serosal) membrane in order to facilitate iron uptake from the blood stream. In addition, basolateral TfR is predisposed to enter into a recycling pathway upon endocytosis, precluding the transfer of receptors to the apical membrane. Transcytosis enhancers have previously been used to overcome this dilemma to enable oral *in vitro* and *in vivo* protein drug delivery (23–25). Transcytosis enhancers were thus considered a means to enable Tf carrier-mediated drug delivery at the pulmonary epithelium. BFA and T-8 have previously been used as transcytosis enhancers. In contrast to previously studied systems (25), T-8 proved to rapidly destabilize the AEC monolayer through unknown mechanisms, precluding the study of TfR-mediated transcytosis (data not shown), while cellular tight junctions were maintained throughout the experiment in the presence of BFA. Our efforts therefore focused on the use of BFA as an enhancer of TfR-mediated transcytosis across alveolar epithelium.

BFA was found not to significantly alter the polar distribution of TfR in type II cell-like AEC monolayers (i.e., KGF-treated), as measured by binding of ^{125}I-Tf at 4°C (Fig. 1B). However, BFA treatment did enhance uptake of ^{59}Fe-Tf from the apical membrane and concomitantly decreased up-

take of ^{59}Fe-Tf from the basolateral membrane of type II cell-like AEC monolayers (Fig. 1D). This apparent discrepancy can be resolved through the following hypothesis. BFA treatment is postulated to induce missorting of TfR, derived from the basolateral endocytic-recycling pool or from nascently synthesized receptor, such that a portion of the TfR is misrouted to the apical membrane where the receptor is only fleetingly situated prior to being resorted back to the Golgi complex. In support of this hypothesis, it is useful to note that BFA has previously been shown to reversibly alter the cis- and trans-Golgi complexes and to disrupt normal endoplasmic reticulum-Golgi interactions (26–28), which would tend to support the possibility that TfR could be missorted to the apical membrane. In addition, we have previously demonstrated a similar result when Madin-Darby canine kidney (MDCK) cell monolayers are treated with BFA (23). The MDCK cells also exhibited similar behavior in response to BFA treatment, with rate of uptake increased at the apical membrane without any change in the average number of receptors present at the apical membrane (23). The MDCK cell line used exhibits high TEER (approx. 2500 Ωcm^2), characteristics similar to the primary AEC monolayers used in the current study. Lastly, the fact that the amount of TfR residing at the basolateral membrane was slightly diminished upon BFA treatment (Fig. 1B) in type II cell-like monolayers, and that the level of TfR endocytosis from the basolateral membrane was also diminished (Fig. 1D), suggests a possible receptor source-pool for the increase in TfR endocytosis from the apical membrane. It is likely that a portion of internalized basolateral TfR would be subject to misrouting to the apical membrane. Even though the 4°C ^{125}I-Tf binding studies were unable to demonstrate a change in static apical TfR distribution in response to BFA treatment, the ability of BFA to increase uptake of ^{59}Fe-Tf from the apical surface suggested a possible means to facilitate apical-to-basolateral transcytosis in type II cell-like AEC monolayers.

Our results indicate that BFA is able to specifically enhance apical-to-basolateral TfR-mediated Tf transcytosis in AEC monolayers of the type II cell-like phenotype (+KGF, Fig. 2A), but not in AEC monolayers of the type I cell-like phenotype (–KGF, Fig. 2B). This result contrasts with previously published reports that indicated enhancement of TfR-mediated transcytosis in AEC monolayers presumed to be of the type I cell-like phenotype (29). AEC monolayers used in our study had much higher TEER values than those in the previous report (approx. 2500 Ωcm^2 compared to approx. 1300 Ωcm^2), suggesting a possible difference in monolayer composition. Our results are not surprising when one considers that type II cells are postulated to be much more metabolically active, playing a critical role in synthesizing and secreting lung surfactant and surfactant proteins, as compared with the relatively quiescent type I cells, whose major biologic roles include formation of the air–interface barrier and fluid clearance from the alveolar spaces, and as such would be more likely to have a much lower iron requirement as compared to type II cells.

Our previous results demonstrated that apical-to-basolateral transcytosis of an insulin-Tf conjugate across Caco-2 monolayers was enhanced by BFA treatment (24). In addition, BFA enhanced the *in vivo* hypoglycemic effect of the insulin-Tf conjugate when administered to streptozotocin-induced diabetic rats (25). Our current results extend these findings to show that the TfR-mediated transcytosis pathway is not limited to Tf conjugates covalently linked to small protein drugs like insulin, but that it can also be used with much larger protein drugs like G-CSF. Since the molecular weight of GCSF (19 kDa) is rather ubiquitous for cytokines in general, this pathway could hold promise for many more protein drugs of interest.

We observed that a covalently linked GCSF-Tf conjugate is able to be transcytosed across type II cell-like AEC monolayers in a manner almost identical to that observed for Tf itself (Fig. 3A). This not unexpected, since BFA-derived enhancement of transcytosis is specific for TfR and does not influence the non-specific transport of other proteins. In support of this statement, Fig. 3B demonstrated that transport of ^{125}I-GCSF in type II cell-like AEC monolayers was not influenced by the presence of BFA. Indeed, GCSF had a substantially higher transport across the AEC monolayer when covalently linked to Tf and subjected to BFA exposure.

In addition to enhancement of transport, TfR-mediated transcytosis of the conjugate appears to deliver the protein drug to the receiver compartment in an unaltered native state. We demonstrated that the molecular weight of the conjugate was not altered when transcytosed via TfR-specific processes (Fig. 4). A minor fraction of degradation products were noted in the chromatographic analyses, most likely due to the routing of a small percentage of the TfR enriched endosomes to the lysosomal pathway. In addition, the protein drug retained biologic activity when released from the conjugate via reductive chemistry (Fig. 5). Our previous studies indicated that free insulin is released from an insulin-Tf conjugate both *ex vivo* (when incubated with rat liver) and *in vivo* (as measured by human insulin radioimmunoassay) (30). The GCSF-Tf conjugate used in this study uses similar disulfide linker chemistry, and it is postulated that GCSF-Tf will be reduced in a likewise fashion *in vivo*. In addition, the lack of substantial biologic activity for the conjugate does not necessarily obviate the potential for the monomer conjugate to retain biologic activity. The current disulfide linker chemistry results in formation of higher-ordered cross-linked products that might potentially be responsible for the lack of biologic activity seen for the conjugate in this study. Optimization of the cross-linking process could result in formation of conjugate products that retain substantial biologic activity. These data indicate that the conjugate is not degraded as a result of TfR-mediated transcytosis and that the GCSF retains biologic activity. Reduction of the labile disulfide bonds within the linker moiety liberates free active protein drug from the conjugate. This reduction phenomenon could be predictive of *in vivo* conjugate reduction characteristics.

In summary, we have demonstrated that TfR is expressed in a polarized fashion in monolayers of the AEC type II cell-like phenotype. In addition, it is proposed that BFA exposure results in transient localization of TfR to the apical membrane with subsequent rapid internalization of these missorted receptors. This was evidenced by the substantial increase in ^{59}Fe-Tf uptake at the apical surface of these monolayers in response to BFA treatment, with the net number of receptors remaining unchanged at the apical surface (4°C, ^{125}I-Tf binding studies). BFA also enhanced TfR-mediated transcytosis of ^{125}I-Tf and the ^{125}I-GCSF-Tf conjugate in the apical-to-basolateral direction across the monolayers. The conjugate was not degraded by this transport process and the

conjugated protein retained biologic activity when recovered from the receiver compartment. This study suggests the possibility of using TfR-mediated transcytosis for systemic delivery of therapeutic proteins via the alveolar epithelium.

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