Transport across a polarized monolayer of Caco-2 cells by transferrin receptor-mediated adenovirus transcytosis

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Abstract

Adenoviral vectors have a poor record of transgene delivery efficiency through physical barriers such as the epithelium or endothelium. We report here the construction of an adenoviral vector that has the capability to be transported across polarized epithelial monolayers of Caco-2 cells (a colon carcinoma cell line) by transcytosis. This transcytosis is transferrin receptor (TfR)-mediated with use of a bifunctional adaptor, soluble coxsackie adenovirus receptor (sCAR)-Tf, and is both temperature and iron dependent. Under experimental conditions, the adenoviral transcytosis was inhibited by pretreatment of Caco-2 cells with colchicine, an inhibitor of transcytosis, and was not enhanced by pretreatment with Brefeldin A (BFA), an enhancer of transcytosis. In these Caco-2 cells, the transcytosis rate was 0.3 ± 1.3% (SD). The transcytosed adenoviruses remain biologically functional. These data suggest the potential clinical benefit under conditions where drug delivery is a challenge, such as within the airway epithelium, at the bladder lumen urothelial cell interface, and across the blood–brain barrier for clinical treatment of lung, urogenital, and brain disorders, respectively, by adenoviral transcytosis of transgene delivery.

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Introduction

Adenoviral vectors are widely used in gene delivery because they have the capacity to infect a broad range of different cell types. Recent studies have identified a 46-kDa host cell membrane protein, coxsackie adenovirus receptor (CAR), that serves as the primary receptor for Ad serotypes 2 and 5 as well as coxsackie B virus attachment to cells (Bergelson et al., 1997; Tomko et al., 1997). Interaction of Ad with a second receptor, αvβ3 integrin, facilitates virus internalization into these same cells (Wickham et al., 1993). However, significant challenges to the efficient use of Ad vectors for in vivo gene delivery exist, including the fact that transgenes are difficult to deliver via Ad vectors into airway lumina (Pickles et al., 1998), at bladder lumen urothelial cell interfaces (Chester et al., 2003), and across the blood–brain barrier (Butt et al., 1990; Crone and Olesen, 1982) because of epithelial and endothelial cell barriers. These lining cells, which are joined together by complex tight intercellular junctions, form a continuous wall against the passive paracellular movement of substances. This is an obstacle for successful gene therapy for the treatment of many disorders including neoplasia.

Receptor-mediated transcytosis can be utilized as a pathway for achieving specific delivery of protein moieties and their peptide constituents across cellular barrier such as the endothelium or epithelium (Shah and Shen, 1996; Tan et al.,...
The polymeric immunoglobulin receptor (pIgR), an integral membrane protein, is required for transcytosis of polymeric immunoglobulin (pIg) including IgA and IgM across mucosal epithelial cell barriers (Mostov and Kaetzel, 1999). After synthesis by plasma cells, polymeric IgA and IgM are commonly in the lamina propria of the mucosal membrane; they bind covalently to pIgR expressed on the basolateral surface of the epithelial layer. The pIg–pIgR complex is then transported via a series of endosomal compartments across the epithelium to the mucosal surface, where it is released as mucosal secretions (Song et al., 1994; Zhang et al., 2000). Another example of transcytosis relevant to the proposed model vector is transferrin (Tf)–transferrin receptor (TfR)-mediated iron uptake (Kishimoto and Tavassoli, 1987; Ma et al., 2002; Roberts et al., 1992). Iron is essential for almost all organisms, fulfilling a variety of biological functions. In human, defects in iron absorption and utilization lead to iron deficiency, overload disorders, and certain neurodegenerative disorders (Andrews, 1999; Lieu et al., 2001). The disease is characterized by inappropriate transmission across an intact epithelial barrier (Banks et al., 1997). HFE is the product of the gene that was transported to the cytosol by DMT1, the iron transporter, resulting in excessive accumulation of iron in organs such as the liver, heart, and pancreas, eventually leading to multi-organ dysfunction (Anderson, 1996). In the intestine, transcytosis of TfR appears to be important in iron homeostasis via regulation of iron regulatory proteins (IRPs) and iron-responsive elements (IREs) at the posttranscriptional level (Hentze and Kuhn, 1996; Klausner et al., 1993; Theil, 1994). The TfR has been found in blood cells, hepatocytes, intestinal cells, monocyte, brain, the blood–brain barrier, and also some insects and bacteria (Lonnerdal and Iyer, 1995; Schryvers et al., 1998). The major pathway for cellular iron uptake is through internalization of the complex of iron-bound Tf:TfR-HFE (Feder et al., 1998; Gross et al., 1998; Parkkila et al., 1997). HFE is the product of the gene that was designated HLA-H that is mutated in >80% of hereditary hemochromatosis patients. Iron is released from transferrin as the result of the acidic pH in the endosome and is then transported to the cytosol by DMT1, the iron transporter (Canonne-Hergaux and Gros, 2002; Garrick et al., 2003; Lam-Yuk-Tsung et al., 2003). The iron is then utilized as a cofactor by heme and ribonucleotide reductase or stored as ferritin. Interestingly, transferrin has been thought of as a “delivery system” with many benefits and has been widely applied as a targeting ligand in the active targeting of anticancer agents, proteins, and genes to primary proliferating malignant cells that overexpress transferrin receptors (Friden, 1994; Quick et al., 2000; Singh, 1999).

The idea of viruses being transported across barriers is feasible. Recent studies suggest that the transcytosis of HIV by epithelial cells plays an important role in HIV transmission across an intact epithelial barrier (Banks et al., 2001; Hocini et al., 2001). A group from Taiwan reported that Japanese encephalitis virus is transported across the cerebral blood vessels by endocytosis in mouse brain (Liou and Hsu, 1998). However, there have been no publications to date describing adenovirus transcytosis via either pIgR or TfR. We expect that if an adenoviral vector could cross epithelial or endothelial cells by transcytosis, it would be beneficial for gene delivery clinical applications.

In this study, (1) Caco-2 cells, which overexpressed transferrin receptor, were used as targeting cells; (2) a fusion protein, soluble CAR (sCAR)-Tf, was used as a bifunctional adaptor; and (3) an adenoviral vector, AdGL3BCMVLuc, which is a non-replicative adenovirus containing the luciferase reporter gene driven by a cytomegalovirus (CMV) promoter, was used as a vehicle for examining cellular transport across a polarized monolayer. The data showed that this adenoviral vector construct is transported across Caco-2 cells by transferrin receptor-mediated transcytosis. Further, the data demonstrated that transcytosed adenoviruses after crossing the polarized monolayer of Caco-2 cell remained biologically functional. This promising evidence of gene delivery across the epithelium by transcytosis via adenoviral vectors has potential significant clinical application in human cancer gene therapy.

**Results**

**Design and generation of the fusion protein, sCAR-Tf**

As discussed in the Introduction, a bifunctional adaptor was used to facilitate Ad transcytosis into epithelial cells in this study. This bifunctional adaptor has two functional domains. One binds to adenovirus, the other to the receptor on the cell surface. It allows adenovirus entry into epithelial cells through a transcytosis response receptor, transferrin receptor (TfR), bypassing coxsackie adenovirus receptor (CAR), the native receptor used by Ad serotypes 2 and 5 for attaching to the cell surface. A fusion protein construct for the sCAR-EGF protein was used as a backbone, and we successfully replaced the region encoding the EGF protein by a full-length cDNA of human transferrin, which was amplified from a human transferrin cDNA clone, pTIR27A. The sCAR-Tf fusion protein clone was termed pFBsCAR6hTf (Fig. 1A) and contains a pPolh promoter, a human CAR ectodomain coding region, six histidine tag, a short flexible linker (SASASAPGS), 2040 bp of human transferrin cDNA, and a poly-A signal. The internal six His tag was used for rapid and efficient purification via immobilized metal ion affinity chromatography, Ni-NTA Sepharose as described in Materials and methods. A short flexible linker was used for effectively separating the two domains of the bifunctional fusion protein. To produce sCAR-Tf, recombinant baculoviruses containing the genes of interest were created and used to infect cells. Infection of High Five cells with this recombinant baculoviruses resulted in a high level of sCAR-Tf expression in both cells and supernatant.
Analysis of the sCAR-Tf protein

The proteins produced with the baculovirus system were verified by Western blotting and the results shown in Fig. 1B. In panel I, a primary antibody, mAb anti-sCAR, and goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) were used. The sCAR-Tf protein was detected both in the cell lysate and in the
His6-tagged proteins expressed in insect cells were purified on a Ni-NTA-Sepharose column in cell lysate or supernatant postinfection days 2, 3, and 4, and tagged with His6. (B) Analysis of the presence of recombinant sCAR-Tf protein by Western blotting. Soluble CAR-His6- and sCAR-Tf terminus of the CAR domain (238 aa). To construct the sCAR-Tf protein, human Tf (679 aa) was fused with the CAR ectodomain by a flexible linker baculovirus expression vector. Expression is driven from the polyhedrin promoter (pPolh). A His6 tag was introduced for purification purposes into the carboxy...

Fig. 1. Characterization of sCAR-Tf fusion proteins. (A) Construction of the fusion protein sCAR-Tf. The gene coding for sCAR-Tf was constructed in a baculovirus expression vector. Expression is driven from the polyhedrin promoter (pFpolh). A His6 tag was introduced for purification purposes into the carboxy terminus of the CAR domain (238 aa). To construct the sCAR-Tf protein, human Tf (679 aa) was fused with the CAR ectodomain by a flexible linker (SASASASAPGS) and tagged with His6. (B) Analysis of the presence of recombinant sCAR-Tf protein by Western blotting. Soluble CAR-His6- and sCAR-Tf-His6-tagged proteins expressed in insect cells were purified on a Ni-NTA-Sepharose column in cell lysate or supernatant postinfection days 2, 3, and 4, and analyzed by electrophoresis on a 12% polyacrylamide gel under denaturing conditions. In panel I, the mAb anti-sCAR was used as the primary antibody; in panel II, mAb antihuman transferrin was used as the primary antibody. A goat anti-mouse IgG conjugated to horseradish peroxidase was used as a second antibody in both panels I and II. The negative controls included the protein purified from uninfected insect cells and PBS. Numbers on the left sides indicate molecular masses of marker protein in kilodaltons. (C) Analysis of the interaction between recombinant protein, sCAR-Tf, and a series of increasing doses of iron (0, 0.1, 1, 10, 100 μM, or 1 mM) at 37 °C for 15 min in 20 μl PBS was given to verify the role of iron during internalization of the Ads. The results showed (Fig. 3) that iron was not essential, but rather was related to the binding between Tf and TfR. Endocytosis mediated by Tf–TfR reached a level 480,000 RLU in the absence of iron (in lane 1). However, although 10 μM of iron concentration almost doubled the internalization level of Ads to 830,000 RLU (in lane 4), this response was lost at higher doses of iron exposure (lane 5 and 6).

Internalization of adenovirus via transferrin receptor on Caco-2 cell surfaces

AdGL3BCMVLuc contains a luciferase reporter gene driven by the CMV promoter and is a non- Replicate adenoviral vector, because the gene (E1) related to virus replication was deleted. When Caco-2 cells were infected with Ads, the expression level of the luciferase reporter gene corresponded to the Ad level at entry into the cells. We, thus, measured the luciferase activity from the cell lysate to evaluate the amount of Ad entry into the cells through the transferrin receptor compared to the native CAR receptor. The data shown in Fig. 2A. In lane A, Ad infection was driven by the CMV promoter into Caco-2 cells via CAR and set at 100% relative light units (RLU). In lane B, Ads were dramatically less able to infect Caco-2 cells when the CARs were blocked with 5 μg/ml of Ad5knob (0.3% RLU compared to lane A). In lane C, the Ads entered the cells by the bifunctional adaptor sCAR-Tf: the luciferase level was lifted to 1.2% as compared to lane A. In lane D, the conditions were identical as in C except the sCAR-Tf was preincubated with mAb (1:1000 dilution) against human transferrin. Luciferase activity was seen to be reduced to 0.8% compared to lane A. This indicates that the endocytosis of adenovirus was partially inhibited by the mAb with an inhibition rate of 33.3%. Because mAb antihuman transferrin did not efficiently inhibit the binding between the adaptor, sCAR-Tf, and TfR, and did not abolish the internalization of Ad, we used the apo-transferrin protein to block TfR. The results are shown in Fig. 2B. Apo-transferrin efficiently inhibited internalization of Ads into Caco-2 cells at an inhibition rate of 70% when the dose reached 6 μg/ml of transferrin, and the inhibition curve was dose responsive to apo-transferrin protein. The data verified Ad internalization into Caco-2 cells did occur through TfR when the native CAR pathway was blocked.

Iron effects on binding of Tf to TfR

The fundamental role of transferrin is to control the levels of free iron (Fe2+) in body fluids by binding, sequestering, and transporting Fe3+ ions, which helps to maintain the availability of iron and prevent the deposition of insoluble ferric hydroxide aggregates. Iron binding to Tf may maintain the conformation of Tf needed for its interaction with TfR. To test this hypothesis, 108 vp AdGL3BCMVLuc was preincubated with 500 ng of the fusion protein sCAR-Tf, and a series of increasing doses of iron (0, 0.1, 1, 10, 100 μM, or 1 mM) at 37 °C for 15 min in 20 μl PBS was given to verify the role of iron during internalization of the Ads. The results showed (Fig. 3) that iron was not essential, but rather was related to the binding between Tf and TfR. Endocytosis mediated by Tf–TfR reached a level 480,000 RLU in the absence of iron (in lane 1). However, although 10 μM of iron concentration almost doubled the internalization level of Ads to 830,000 RLU (in lane 4), this response was lost at higher doses of iron exposure (lane 5 and 6).
Transcytosis of Ads across a monolayer of Caco-2 cells

We have demonstrated that the internalization of AdGL3BCMVLuc into Caco-2 cells via TfR on the surface of the cells occurs. We tried to investigate whether free adenoviral particles were capable of transcytosis through the Caco-2-polarized monolayer via TfR with a bifunctional adaptor sCAR-Tf. The data are shown in Fig. 4A. The Ads were preincubated with sCAR-Tf at 37 °C for 15 min, leading to the domain of sCAR of the adaptor binding to the knob of Ad. The mixture was added into the basal chamber of the transwell plate after the cells had previously been blocked with Ad5knob. The medium in the apical chamber was collected at different time points, and the copy number of the Ad E4 gene was detected by quantitative real-time PCR. The copy number of adenoviruses detected in the apical side was increased 500-fold 6 h postincubation. Adenoviruses detected on the apical side were not secondary to leakage of the Caco-2 cell monolayers because the copy number dropped more than 10-fold if the bifunctional adaptor sCAR-Tf was excluded from the transcytosis system. This suggests that adenoviruses transferred to the apical chamber from the basal chamber via a Tf receptor-mediated mechanism.

Also, the transcytosis was temperature related, similar to that noted previously in the literature (Hocini et al., 2001). Fig. 4B shows transcytosis of free viruses was inhibited by decreasing temperature. Twenty percent of transcytosis was inhibited by incubating at 15 °C, and more than 90% was inhibited at 4 °C. The possibility that the level of transcytosed Ads at 4 °C incubation might represent background leakage of the polarized monolayers of Caco-2 cells cannot, however, be completely excluded.

Enhancement and inhibition of transcytosis

An experiment was performed to further verify adenovirus transcytosis in a Caco-2-polarized monolayer via TfR. We set the transcytosis rate as 100% in the transcytosis group (Lane A) in which CAR on the cell surface was blocked with Ad5knob, and the AdGL3BCMVLuc was pretreated with bifunctional adaptor sCAR-Tf. Seventy percent and 90% of transcytosis rate of Ads, respectively, were inhibited when TfR was blocked with either human apo-transferrin (6 μg/ml) (lane D) or the Ads were without the bifunctional adaptor sCAR-Tf (lane F). The data further verify that the transcytosis of adenoviruses in a Caco-2-polarized monolayer is TfR-mediated. Transcytosis rates in Caco-2 cells were also partially inhibited by treatment with iron.

Transcytosis of Ads across a monolayer of Caco-2 cells

![Graph showing transcytosis rates](image-url)

**Fig. 2.** Endocytosis of adenoviruses into Caco-2 cells via TfR. (A) Endocytosis of AdGL3BCMVIinto Caco-2 cells was detected by luciferase activity reported as relative light units (RLU). Lane A, 10⁵ vp of AdGL3BCMVI was added to the basal chamber only; lane B, before adding 10⁵ vp of AdGL3BCMVI to the basal chamber, CARs on the cell surface were blocked with Ad5knob; lane C, same as lane B, the AdGL3BCMVI was pretreated with 500 ng of sCAR-Tf as described in the Materials and methods; lane D, antihuman transferrin mAb was used to block the Tf domain in the fusion protein. Lane A was set as 100% RLU. (B) Inhibition curve of adenovirus endocytosis through a tight Caco-2 epithelial barrier by a human apo-transferrin protein. The CAR-blocked Caco-2 cells were incubated with increasing amounts of apo-transferrin before adding the mixture of Ads and sCAR-Tf.

![Graph showing inhibition of transcytosis](image-url)

**Enhancement and inhibition of transcytosis**

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![Graph showing iron effect on Tf binding](image-url)

**Fig. 3.** Iron effect on Tf binding to TfR. AdGL3BCMVI was pretreated with the fusion protein sCAR-Tf and exposed to an increasing amount of iron (FeCl₃, 0, 0.1, 1, 10, 100, and 1000 μM). Then the mixture was added to the basal chamber and the luciferase activity was measured as RLU in the cell lysate after 24-h incubation.
Colchicine (32%) and antihuman Tf mAb (34%) compared to lane A, respectively. However, it was not evident that the transcytosis of adenovirus was enhanced by pretreating cells with 1.6 $\mu$g/ml Brefeldin A (BFA, lane B), which was previously reported to have been seen during TfR transcytosis.

**Adenovirus biological function after transcytosis**

Cellular uptake of iron from transferrin occurs via transferrin receptor-mediated endocytosis. Diferric transferrin is bound to the membrane-bound transferrin receptor and internalized via receptor-mediated endocytosis into endosomes. Upon acidification (pH 5.5), iron is released and then transported by the iron transporter DMT1 into the cytoplasm. To verify whether the acidification (pH 5.5) during transcytosis destroys transcytosed Ads or affects their biological function, we collected the medium from the apical chamber after adding AdGL3BCMVLuc into the basal chamber and incubating for 6 h at 37 °C. The medium was used to infect 293 cells as described in Materials and methods. After 24-h incubation at 37 °C, luciferase activities were measured. The luciferase activity in the transcytosis group, in which the Ads were pretreated with bifunctional adaptor (sCAR-Tf), was much higher ($8 \times 10^6$ RLU ± SD, $n = 6$) than that in the non-transcytosis group with a significant difference ($P < 0.01$). The data demonstrate that the acidification in endosomes does not affect the function of the transcytosed adenoviruses during transportation from the basal chamber into the apical chamber via a Tf–TfR interaction.

**Discussion**

Adenoviral vectors possess several attributes that render them useful gene delivery vehicles for systemic gene therapy. In particular, the in vivo transduction efficiencies achievable with these agents are greater than with currently available alternative vector system (Akusjarvi, 1993). However, the efficacy of delivery of adenoviral vector passing through the epithelium and endothelium is very low due to the physical barriers described above. The purpose of these studies was to characterize the ability of adenoviruses to cross epithelial cells as free viruses. The use of the Ad vector AdGL3BCMVLuc has a distinct advantage for this purpose. First of all, this vector is a non-replicative virus because the response gene (E1) for virus replication was deleted. The Ad E1 gene contains E1 A and E1B genes; E1 A proteins act as major regulators of early viral transcription and proliferation (Akusjarvi, 1993; Dyson, 1998), and E1B proteins act as inhibition of apoptosis and DNA replication (Chinnadurai, 1998; White, 1998). This viral vector can only be replicated in cell lines in which the E1 gene is complemented, such as the 293 (Graham et al., 1977) or 911 (Fallaux et al., 1996) cell lines. Non-replicative viruses make it possible to quantitatively measure the amount of viruses for endocytosis or transcytosis without viral amplification in cells. Second, this vector contains a reporter gene, the luciferase gene, that is driven by a strong CMV promoter (Yamamoto et al., 2001). The luciferase activity should be easily and sensitively measured by a commercial kit even if the Ad of endocytosis is present in trace amounts. Third, when the luciferase gene has not yet to be expressed due to a short incubation time (such as less than 6 h), the copy number of Ads can be detected by performing an E4 gene assay using a quantitative real-time PCR technique.

The transcytosis of epithelial or endothelial cells requires two major receptors previously described, plgR and TfR. The human colon adenocarcinoma-derived cell line Caco-2 has the components currently known to be required for iron
transport, including TfR, DMT1, hephaestin, ferroportin1, and HFE (Ma et al., 2002). These cells form a polarized monolayer when grown on a semiporous membrane, allowing demonstration of the transcytosis of adenoviruses from the basal to the apical chamber. However, the native receptor of adenoviruses CAR was also highly expressed on the cell surface of Caco-2 cells (data not shown). Therefore, it was important in the demonstration of transcytosis to block CAR completely on the cell surface by use of Ad5knob. The data shown here indicate that the entry of adenoviral vectors was inhibited more than 99% when the CARs on cell surface were blocked with Ad5knob (Fig. 2A).

The technical achievement of Ad retargeting via bispecific molecular complexes has been approached by a variety of methods, using folate (Kranz et al., 1995), epithelial growth factor, EGF (Dimitriev et al., 2000), c-erb B-2 oncoprotein (Kashentseva et al., 2002), and α6 integrin protein (Wickham et al., 1996). In this regard, abrogation of Ad native tropism was achieved by the use of the anti-knob antibody or sCAR conjugated with ligands specific for the receptor on the target cell surfaces. The advantage of this technique is that adenoviruses retarget to a new site on the surface of cells as well as ablate the native tropism of Ad. We designed a bifunctional adaptor sCAR-Tf that should ablate the native tropism of Ad with the sCAR domain and efficiently retarget Ad to TfR on the cell surface with a Tf domain. This fusion protein was successfully expressed in High Five cells and purified by Ni-nitrilotriacetic acid (NTA)-Seaphorose. Un-mature fusion protein purified from cell lysates had a much poorer binding function with either Ad knob or with TfR compared to the mature fusion protein, which was purified from the supernatant. It was clear that the molecular mass of the fusion protein from the supernatant is bigger than that from the cell lysate (Fig. 1B) because of glycosylation. Glycosylation modifications of the protein are known to play an important role in maintaining the function of the protein. As previously reviewed, N-glycosylation is required for full activity of the murine galactosylceramide sulphotrasferase (Eckhardt et al., 2002), and Chakrabarti et al. (2002) reported that modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. It is possible the glycosylation of the fusion protein sCAR-Tf in the Golgi network allows the fusion protein to have a biological secondary structure for both domains that thus enhance their binding function.

Endocytosis of adenovirus was observed in Fig. 2A, although the ratio for Ad endocytosis into Caco-2 cells is relatively modest, only 1.2% in lane C. To verify that endocytosis is transferrin receptor-mediated, we treated the fusion protein with mAb anti-Tf for blocking the binding between Tf and TfR. The data shown in Fig. 2A, lane D, indicate that only 30% of endocytosis was inhibited by pretreatment of the fusion protein with mAb anti-Tf. However, the endocytosis was inhibited 70% by pretreatment of Caco-2 cells with apo-transferrin for blocking TfR. The data thus suggest that the endocytosis of adenovirus was transferrin receptor-mediated. Monoclonal antibodies recognize an epitope by few residues, usually 5–10 amino acids. For example, the monoclonal antibody Syn-1 has been frequently used for the specific detection of alpha-synuclein. The epitope for Syn-1 is localized within residues 91–99 of human alpha-synuclein (nine residues) (Perrin et al., 2003). Other reports described that ELDWA (five residues)-epitope-specific monoclonal antibodies inhibit HIV-env-mediated syncytium formation (Zhang and Chen, 2003). We prepared the fusion protein sCAR-Tf with 679 residues of transferrin including the N- and C- terminals that include the key domain for TfR binding (Lawrence et al., 1999). In this regard, we hypothesize that the mAb anti-Tf, whose recognition site to Tf has not yet been found, binds to the epitope on Tf in a configuration that is not close enough to the binding site of TfR that would result in complete inhibition, thus only partial inhibition of the endocytosis is observed.

The transferrin receptor (TfR) assists iron uptake into vertebrate cells through a cycle of endo- and exocytosis of transferrin (Tf) (Richardson and Penka, 1997). The TfR has been found in red blood cells, thyroid cells, hepatocytes, intestinal cells, monocytes, and brain cell elements. In malignant cells, there are elevated levels of TfR expression supporting their growth (Huebers and Finch, 1987). The TfR is also localized on the endothelial surface of brain capillaries that compose the blood–brain barrier (Jefferies et al., 1984). Generally, transferrin receptors have a higher affinity to diferric transferrin than apo-transferrin (Sun et al., 1999). In our data (Fig. 3), the binding affinity was elevated by low concentrations of iron (the range from 0.1 to 10 μM), but inhibited at high concentrations (100–1000 μM). The binding between sCAR-Tf and TfR was detected to a certain level even if the iron lacked in this system. Therefore, it seems that binding between Tf's and TfR benefits from the presence of iron, but this is not essential.

We investigated whether free adenoviruses in the basal chamber were capable of transcytosing into the apical chamber through the polarized monolayer of Caco-2 cells via TfR and a bifunctional adaptor sCAR-Tf. The data show in Fig. 4A a significant amount of transcytosis was consistently observed during incubation times from 30 min to 6 h. At 6 h, the recovery rate in the apical chamber as measured with real-time PCR for the Ad E4 gene was 0.3 ± 0.13% (mean ± the standard error of the mean). This ratio is very similar to the data published by Hocini et al. (2001) in which the ratio range was from 0.01 ± 0.005% to 0.77% ± 0.16% for recovery rates of transcytosis of different HIV-1 isolates through a tightly polarized monolayer of human endometrial cells. The transcytosis was decreased more than 10-fold if the system contained no bifunctional adaptor. This is strong evidence that the transcytosis is TfR-mediated via the bifunctional adaptor sCAR-Tf. Compared to the level of transcytosis Ads (292 E4 gene copy number), the amount of
endocytosis Ads (2850 E4 gene copy number) was about 10-fold higher at the 6-h point. In this regard, Sheff et al. (1999) demonstrated that the majority of transferrin (65%) was found to be recycled from the early endosomes within 10 min after internalization in an MDCK cell model. This suggested that most of internalized Ads may have been recycled directly back to the basolateral membrane and thus allowed only a small amount (0.3% in this study) of Ads across to the apical chamber via transcytosis.

Brefeldin A (BFA) is an agent described to cause an increase in TfR transcytosis. Shah and Shen (1996) demonstrated a combination of conjugated insulin and BFA can cause a net 45-fold increase in apical-to-basolateral transport of insulin across Caco-2 cell monolayers. Wan et al. (1992) showed data that BFA (1.6 μg/ml) markedly enhanced the transcytosis of 125I-labeled Tf (125I-Tf) in both apical-to-basal and basol-to-apical directions. In our system, we did not find the enhanced effect of adenovirus transcytosis in filter-grown Caco-2 cell monolayers (Fig. 5, lane B). These data are similar to that described for transcytosis of albumin in endothelial cells in a Brefeldin A-independent manner as described by Antohe et al. (1997). Similarly, BFA did not enhance the transcytosis of either native horseradish peroxidase (HRP) or membrane-bound HRP-poly (L-lysine) conjugates in a Caco-2 cell monolayer as described by Wan et al. (1992). Colchicine treatment of Caco-2 cells for 4 h depolymerized 99.4% of microtubular tubulin in studies reported by Gilbert et al. (1991). In our experiments as shown in Fig. 5, lane C, transcytosis was inhibited 30% by the irreversible depolymerization of microtubules with colchicine. This argues that transcytosis by adenovirus in polarized monolayers of Caco-2 cells is partially mediated by the active movement of the cytoskeleton.

Most, if not all, mammalian cells are capable of taking up iron by receptor-mediated endocytosis of diferric transferrin bound to the transferrin receptor. This process is complex and can be divided into seven steps, that is, (1) binding, (2) internalization (endocytosis), (3) acidification, (4) dissociation, (5) reduction, (6) translocation, and (7) cytosolic transfer of iron into intracellular compounds such as ferritin and heme (Aisen, 1998; Qian and Tang, 1995). Cellular uptake of iron is initiated by the binding of diferric-transferrin to a specific TfR on the outer face of the plasma membrane. In fact, it has been demonstrated that HFE, the protein mutated in hereditary hemochromatosis, and Fe3+-Tf can bind simultaneously to TfR to form a ternary complex, Tf–TfR–HFE. After endocytosis via clathrin-coated pits, which eventually bud from the plasma membrane as membrane-bound vesicles or endosome, the Tf–TfR–HFE complexes are routed into the endosomal compartment. Upon maturation and loss of the clathrin coat, the endosome becomes competent to pump protons in a process energized by adenosine triphosphate (ATP) and the endosomal lumen is rapidly acidified to a pH of 5.5 (Dautry-Varsat et al., 1983; Klausner et al., 1983). Iron is released from Tf. The acidification has been thought to be essential for the efficient release of iron. This process should be the same for the transfer and release of adenoviruses by transcytosis in the Caco-2 cell model. It is important to determine if the acidification in the endosome has the capacity to destroy or disassemble the transferred adenoviruses or not. We detected luciferase activity expressed by the AdGL3BCMV vector in 293 cells in the apical chamber (Fig. 6). These data verified that the transcytosed adenovirus retains its infectious activity, is capable of infection of 293 cells, and expressed the reporter gene luciferase in 293 cells (Fig. 6, lane A). The luciferase activity was inhibited when the transcytosis system contained no bifunctional adaptor sCAR-Tf (Fig. 6, lane B), yielding a significant statistical difference (P < 0.01). Again, the data verified that the transcytosis of adenovirus was Tf–TfR-mediated.

Our data suggest that adenoviruses can be transferred to an apical chamber from the basal chamber via transcytosis, which is TfR-mediated and bifunctional adaptor-associated. Whereas our study has clearly established the feasibility of exploiting transcytosis to achieve transition of a cellular barrier, the magnitude of the effect remains limiting. In this regard, transcytosis rates may be linked to the intrinsic recycling efficiency of a given pathway. On this basis, exploiting alternate transcytosis pathways such as P1gR may allow an enhancement in the functional transcytosis of Ad. In addition, for a given pathway, transcytosis rates could also be augmented by maneuvers to increase Ad-
target receptor binding via augmentations in target receptor levels or enhancement in the affinity between Ad-incorporated targeting ligand and the target receptor. It is also noteworthy that Ad capsid modification contributes to particle fate within cellular endosomes. On this basis, it should be possible to allow intracellular fate to achieve the goal of enhanced overall transcytosis.

Materials and methods

Cells

Caco-2 cells (American Type Culture Collection, Rockville, MD; passage number 20) were grown on 0.4-μm pore size polycarbonate filters in transwell chambers (Costar, Cambridge, MA). Cells were grown at 37 °C in medium recommended by the suppliers in a humidified atmosphere of 5% CO₂. Monolayers that displayed a transepithelial electrical resistance (TEER) of 200–300 V/cm² as measured with an epithelial volt/ohm meter (EVOM, World Precision Instruments, West Haven, CT) were used in the experiments.

Before the experiments, cell monolayers were rinsed once with serum-free Dulbecco medium containing 1% nonessential amino acids and 1% L-glutamine. This rinse step was followed by incubation at 37 °C for 1 h with serum-free Dulbecco medium containing 2 mg/ml bovine serum albumin to deplete endogenous transferrin (Tf). This pretreatment did not affect the TEER of the cell monolayer.

Chemicals and enzymes

Ferric chloride, human apo-transferrin, Brefeldin A, and Colchicine were purchased from Sigma (St. Louis, MO) and used in this study. Restriction endonucleases, T4 ligase, and proteinase K were purchased from New England Biolabs (Beverly, Mass.).

Antibodies

A murine monoclonal antibody (mAb 5CA) against soluble CAR (sCAR) protein was a kind gift from Dr. Victor Krasnykh (MD Anderson Cancer Center, Houston, TX). A murine monoclonal antibody against human transferrin protein (mAb α hTf) was purchased from US Biological Corporation (Swampscott, MA).

Viruses

A recombinant Ad5 vector AdGL3BCMVLuc containing a firefly luciferase-expressing cassette in place of the deleted E1 region of the Ad genome was obtained from Dr. Masato Yamamoto (University of Alabama at Birmingham, Birmingham). These viruses had no replicative function because of the deleted E1 gene, which is required for viral replication. Ad was propagated on 293 cells and purified by centrifugation in CsCl gradients by a standard protocol. Virus particle counts were determined spectrophotometrically by the method of Maizel et al. (1968) using a conversion factor of 1.1 x 10¹² viral particles (vp) per absorbance unit at 260 nm. To determine the titer of infectious viral particles, the plaque forming assay on 293 cells was performed by the method of Mittereder et al. (1996).

Construction of recombinant plasmids

To create a construct for expression of the fusion protein sCAR-Tf, the DNA sequence coding for a human transferrin excluding the leading peptide (1–19 amino acids) was amplified from the plasmid pTfR27A, which was purchased from the ATCC, using the primers 5'-GCTAGGATCCGTCCTCCTGATTTAGGAAAGTGCA, and 5'-GCTAGATCCTTAGGAAAGTGCA. Both primers were engineered in a BamHI site (underlined) in a 5' region for cloning. The amplicon encoded the full-length human transferrin cDNA sequence (2040 base pairs (bp)), which was verified by automated sequence data (data not shown) and cloned into the TA clone plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA). A 2040-bp DNA fragment was removed from the Tf-TA clone with partial digestion of the restriction endonuclease BamHI, which eliminated a BamHI site located 272 nt (nucleotides) from the 5' end of the transferrin cDNA.

A fusion protein sCAR-EGF clone pFBsCAR6hEGF was a kind gift from Dr. I. Dmitriev (University of Alabama at Birmingham, Birmingham, AL). It contained a polyhedrin (pPolh) promoter, the human CAR ectodomain cDNA, a six histidine marker, a short linker (SASASASAPGS), human EGF cDNA, and a poly-A signal. The cloning site for human EGF cDNA in pFBsCAR6hEGF was BamHI. We successfully replaced the EGF by human transferrin cDNA.
utilizing the BamHI restriction sites. The new clone was termed pFBsCAR6hTf. After transformation of E. coli DH5α, plasmid clones were sequenced in the region of the insert by using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA). Sequence analysis was performed at the Genomics Core of the Howell and Elizabeth Heflin Center for Human Genetics of the University of Alabama at Birmingham. The resultant plasmid pFBsCAR6hTf was selected. The constructed plasmid encoding recombinant human sCAR fused with human transferrin and tagged with an internal His6 marker that was then used for generation of the recombinant baculovirus genome.

Expression and purification of the histidine six-tagged recombinant fusion protein, sCAR-Tf

The recombinant fusion protein sCAR-Tf was expressed in High Five cells (Invitrogen) infected with the baculovirus by the method recommended by the manufacturer. Briefly, High Five cells were maintained in suspension culture and infected with recombinant baculovirus at an MOI of 10 pfu/cell. The cell suspension was harvested 72–96 h postinfection, and cells were pelleted by centrifugation. Cleared supernatant medium was concentrated 10-fold with a Centricon Plus-20 apparatus (Millipore Corporation, Bedford, MA) and dialyzed against phosphate-buffered saline (PBS). Recombinant proteins were then purified by immobilized metal iron affinity chromatography on Ni-nitrilotriacetic acid (NTA)-Sepharose (Qiagen, Valencia, CA) using both cell pellet and concentrated supernatant as recommended by the manufacturer. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine gamma globulin as the standard.

Western immunoblot analysis

For Western immunoblot analysis, a 300-ng aliquot of sCAR, which was a kind gift from Dr. I. Dmitriev (University of Alabama at Birmingham, Birmingham, AL), and 20 μl of purified fusion protein sCAR-Tf were treated with 10 μl of loading buffer and subjected to sodium dodecyl sulfate (SDS) 12% polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, the protein was transferred onto a Sequi-Blot PVDF membrane (Bio-Rad Laboratories). The membrane was blocked for 1 h at room temperature in TBS (10 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA) containing 5% dried milk and then washed three times, 5 min each, at room temperature with wash buffer (TBS plus 0.1% Tween). The membrane was incubated overnight at 4 °C with either a 1:4000 dilution of a murine monoclonal antibody (mAb 5CA) against sCAR or 1:2000 dilution of a murine monoclonal antibody (mAb α hTf) against human transferrin protein. The membranes were washed three times, 10 min each, at room temperature with wash buffer. Next, the membranes were incubated with a 1:2000 dilution of a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, IL) for 1 h at room temperature. The primary and secondary antibody was diluted with TBS plus 5% dried milk and 0.1% Tween. After washing, the membranes were immersed in Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA) for 5 min at room temperature and then exposed to Kodak X-OMAT film for from 30 s to 5 min.

ELISA

A solid-phase binding enzyme-linked immunosorbent assay (ELISA) was performed to verify the binding function of the fusion protein. Ad5knob was a kind gift from Joshua Short (University of Alabama at Birmingham, Birmingham, AL). The protein was diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6) to a concentration 8.0 pmol/ml, and 100-μl aliquots were added to wells of a 96-well Nunc-Maxisorp ELISA plate. Plates were incubated overnight at 4 °C and then blocked for 2 h at room temperature by the addition of 200 μl of blocking buffer (0.01 M PBS (pH 7.4), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, 2% BSA) to each well. Wells were then washed three times with washing buffer (0.01 M PBS (pH 7.4), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20). Purified fusion protein sCAR-Tf from cell lysate or medium diluted in binding buffer (0.01 M PBS (pH 7.4), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, 0.5% BSA) to a concentration of 50 ng/ml was added to the wells in 100-μl aliquots. After 1 h of incubation at room temperature, the wells were washed three times and bound fusion protein was detected by incubation with 1:2000 dilution of mAb antihuman transferrin (mAb α T.t). Following incubation at room temperature for 1 h, the wells were washed again and incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated with alkaline phosphate (Sigma) for 1 h. The wells were then washed four times, and the plate was developed with p-nitrophenyl phosphate (Sigma) as recommended by the manufacturer. Plates were read in a microtiter plate reader set at 405 nm; results are present as mean ± standard deviation (SD). The tests were performed by excluding the primary mAb as the negative control or background.

Internalization of Ad into Caco-2 cells via transferrin receptor

Caco-2 cells (5 × 10⁴) were plated in 12-well transwell plates in minimum essential medium (Eagle) supplemented with 2 mM t-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 0.1 mM sodium pyruvate, and 20% FBS; and incubated at 37 °C in a 5% CO₂ atmosphere. Basal and apical chambers contained 800 and 200 μl of medium, respectively. When the electric resistance reached 200–300 Ω/cm², the experiments were performed as follow:
(1) Group A, 10^8 vp AdGL3BCMV was added to the basal chamber medium that contained 2% FBS; (2) Group B, before adding AdGL3BCMV, CARs on cell surfaces were blocked with 5 µg/ml of Ad5knob by incubating at 37 °C for 20 min; (3) Group C, CARs were blocked the same as above, 500 ng of sCAR-Tf incubated with 10^8 vp AdGL3BCMV in 20 µl PBS at 37 °C for 15 min was added into the basal chamber; (4) Group D, same as group C, but 1:100 mAb anti-hTf was added into the mixture of sCAR-Tf and 10^8 vp AdGL3BCMV for 15 min at 37 °C to block sCAR-Tf binding to TiR. After adding the Ad into the medium in the basal chamber, cells were incubated at 37 °C overnight. The next day, the polycarbonate filters in the transwell chamber were cut by a razor. Cells on the filters were lysed with cell culture lysis buffer (Promega, Madison, WI), and luciferase activity was measured by the Luciferase Assay System of Promega following the manufacturer’s protocol. Experiments were performed in triplicate and luciferase activities were presented as relative light units (RLU).

**Human apo-transferrin blocks the fusion protein, sCAR-Tf, binding to TiR**

Caco-2 cells (5 × 10^4) were plated in 12-well transwell plates as outlined above. When the electric resistance of the monolayer had reached 200–300 Ω/cm², cells were blocked with 5 µg/ml of Ad5knob and with one of a series of human apo-transferrin (0, 0.6, 2, 6, or 18 µg/ml) doses at 37 °C for 20 min. A mixture of 10^8 vp AdGL3BCMV preincubated with 500 ng of the fusion protein sCAR-Tf was added to the basal chamber medium containing 2% FBS. The luciferase activities of the cells were measured as before. Experiments were performed in duplicate and luciferase activities were presented as the means. The inhibition rates were calculated using the formula: inhibition rate = RLU with apo-transferrin/RLU without apo-transferrin × 100%.

**Iron effect on Tf binding to TiR**

Caco-2 cells (5 × 10^4) were plated in 12-well transwell plates as before. When the electric resistance of the monolayer had reached 200–300 Ω/cm², cells were blocked with 5 µg/ml of Ad5knob and one of a series of iron doses (0, 0.1, 1, 10, 100 µM, or 1 mM) was delivered at 37 °C for 15 min in 20 µl PBS. Then, the mixture was added to the medium containing 2% FBS in the basal chamber. Medium was collected from the apical chamber, and the copy number of the Ad E4 gene was detected by quantitative real-time PCR. Experiments were performed in triplicate and the data presented as the means ± SD.

For transcytosis enhancement or inhibition, filters grown Caco-2 monolayers were incubated with Brefeldin A (BFA, 1.6 µg/ml), present in the basal medium for 5 h at 37 °C as described Wan et al. (1992). Following exposure to BFA, cells were rinsed extensively, and the chambers were refilled with MEM. Alternatively, monolayers of Caco-2 cells were pretreated with 10 µM of colchicine for 30 min at 4 °C followed by a 10-min incubation at 37 °C (Morizono and Harada, 1998). Excess drug was removed by extensive washing. Then, 10^8 vp AdGL3BCMV was preincubated with 500 ng of the fusion protein sCAR-Tf at 37 °C for 15 min in 20 µl PBS and the mixture was added to the medium containing 2% FBS in the basal chamber. Transwell plates were incubated at 37 °C for 6 h. Then, the medium was collected from the apical chamber, and the copy number of the Ad E4 gene was detected by quantitative real-time PCR as described below. Experiments were performed in triplicate and the data were presented as the means ± SD.

Quantitative real-time PCR for E4 detection

Adenovirus DNA samples were collected from the apical medium of the transwell plates. The fluorescent TaqMan probes and the primer pairs used for real-time PCR in analysis of the copy number of adenovirus by detecting the E4 gene were 5’-TGGC ATGACA CACTAC-GACCAACACGATCT (probe), forward primer 5’-GGAGTGC GCCGGAGACAAC, and reverse primer 5’-ACTACGTCGCGCTTCCAT. They were designed using Primer Express 1.0 (Perkin-Elmer, Foster City, CA) and
synthesized by Applied Biosystems. For the real-time PCR assay, each 9 µl PCR reaction contained 3 mM MgCl₂, 300 µM each of dATP, dCTP, and dGTP, 600 µM dUTP, 100 nM of forward and reverse primers, and probe, 1 U of rTth DNA polymerase, 0.025% BSA, and RNase-free water. Two microliters of adenovirus DNA sample was added into each assay tube. Negative controls without template were performed for each reaction series. Real-time PCR reaction was carried out using a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN). Thermal cycling conditions were 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, then 40 cycles of 20 s at 94 °C, and 1 min at 62 °C. Data were analyzed with Light Cycler software.

Ad biological function assay after transcytosis

Caco-2 cells (5 × 10⁴) were plated in 12-well transwell plates as before. When the resistance of the monolayer had reached 200–300 Ω/cm², cells were blocked with 5 µg/ml of Ad5knob at 37 °C for 20 min. AdGL3BCMV (10⁵ vp) was preincubated with or without 500 ng of the fusion protein sCAR-TF at 37 °C for 15 min in 20 µl PBS. Then, the mixture was added to the medium containing 2% FBS in the basal chamber. Four hours postincubation at 37 °C, 200 µl of medium containing transcytosed adenoviruses was collected from the apical chamber and transferred to the 293 monolayer in 24-well plate in infection medium containing 2% FBS. Luciferase activities were measured from the 293 cell lysate after 24-h incubation at 37 °C with a Luciferase Assay Kit (Promega). Experiments were performed in triplicate and luciferase activities were presented as the RLU means ± SD.

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References


