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Binding-incompetent Adenovirus Facilitates Molecular Conjugate-mediated Gene Transfer by the Receptor-mediated Endocytosis Pathway*

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Molecular conjugate vectors may be constructed that accomplish high efficiency gene transfer by the receptor-mediated endocytosis pathway. In order to mediate escape from lysosomal degradation, we have incorporated adenoviruses into the functional design of the conjugate. In doing so, however, we have introduced an additional ligand, which can bind to receptors on the cell surface, undermining the potential for cell specific targeting. To overcome this, we have treated the adenovirus with a monoclonal anti-fiber antibody, which renders the virus incapable of binding to its receptor. The result is a multi-functional molecular conjugate vector, which has preserved its binding specificity while at the same time being capable of preventing lysosomal degradation of endosome-internalized conjugate-DNA complexes. This finding indicates that adenoviral binding is not a prerequisite for adenoviral-mediated endosome disruption.

Gene transfer can be accomplished via the receptor-mediated endocytosis pathway employing molecular conjugate vectors (1–8). This vector system consists of two linked functional domains: a DNA binding domain and a ligand domain that recognizes a specific cell surface receptor. When the ligand domain is recognized by the appropriate cell surface receptor, the conjugate-DNA complex is internalized by the receptor-mediated endocytosis pathway, co-transporting the bound DNA. One of the potential advantages of molecular conjugates is that this vector system possesses the capacity to target specific cells by virtue of the ligand domain of the conjugate. Thus, through the choice of the ligand, it is theoretically possible to specifically target any cell type by virtue of a specific cell surface receptor. In this regard, Wu and coworkers (1, 2) have constructed an asialoglycoprotein-polycation conjugate that has been shown to specifically target hepatocytes both *in vitro* and *in vivo* via asialoglycoprotein receptors found on the surface of this cell type.

Although molecular conjugates possess a specific and efficient internalization mechanism, the fact that they lack a mechanism to accomplish escape from lysosomal degradation after cellular internalization has limited gene transfer efficiency (5, 9). As a strategy to prevent the endosome-internalized DNA from being retained within the cell vesicle (which would limit gene transfer efficiency), we have incorporated both replication-competent and -incompetent adenoviruses into the functional design of the conjugates (10–12). Adenoviruses, which also enter cells via the receptor-mediated endocytosis pathway, possess a specific mechanism to escape the cell vesicle system by mediating disruption of the endosomes (13). When incorporated into the conjugate design, adenoviruses can facilitate entry of the conjugates into the cytoplasm by disrupting the endosome, thus greatly enhancing overall gene transfer efficiency (10, 11).

In this configuration, however, the viral moiety functions in the capacity of both an endosome lysis agent and also as an alternate ligand domain of the conjugate. Thus, since an additional ligand has been introduced into the conjugate design, the potential for cell specific targeting may be undermined. The goal of the present study was to selectively exploit the endosome lysis functions of the adenovirus while concomitantly eliminating the adenovirus as a alternate ligand. In order to accomplish this, a monoclonal antibody to the fiber protein of adenovirus serotype 5 was generated to ablate adenoviral binding. We have shown that when introduced into the conjugate design, antibody-coated virions retain the ability to disrupt endosomes but can no longer function as alternate ligands. Interestingly, the demonstration of selective exploitation of adenoviral endosome lysis establishes that adenoviral binding and vesicle disruption are not functionally linked.

MATERIALS AND METHODS

Cell Lines—HeLa cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM)[1] with 4500 mg/liter glucose and L-glutamine (DMEM-H) supplemented with 5% fetal bovine serum (FBS), penicillin, and streptomycin (complete media). The cell line 293 was grown as a monolayer in DMEM-H supplemented with 10% FBS, penicillin, and streptomycin (high serum media).

Adenovirus—Adenovirus was propagated and purified as previously described (14). The adenovirus P202-Ad5 is a chimeric serotype 5 adenovirus that contains a *Mycoplasma pneumoniae* P1 protein epitope as part of the hexon capsid protein, which is used to immunologically link poly(L-lysine) to the virus (10). The cell line 293 was used for viral propagation. After density centrifugation, purified virions were stored in viral preservation media (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mg/ml bovine serum albumin, and 50% (v/v) glycerol) at −70 °C.

Preparation of ^{35}S-Labeled Virus—Adenovirus labeled with [^{35}S] methionine was produced in HeLa cells as described (15). Briefly, HeLa cells were seeded in 75-cm tissue culture flasks as monolayers 18 h before infection with adenovirus P202. To the HeLa flasks P202-Ad5 (10–100 plaque-forming units/cell), diluted in DMEM-H supplemented with 2% FBS, penicillin, and streptomycin (low serum media), was added. The flasks were then incubated for 1 h at 37 °C, after which time complete media was added. The infection was allowed to proceed for 16 h, at which time the media was removed and replaced by medium containing methionine-free DMEM, 5% dialyzed fetal

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum, mAb, monoclonal antibody; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; hTfpL, human transferrin-poly(L-lysine); hTfpL/AdpL, ternary complex.

calf serum, penicillin, streptomycin, and 5 mCi of [^{35}S]methionine (Amersham Corp.) for a 48-h period. Cells were then removed from the plastic surface by vigorous shaking, and the cell suspension was centrifuged at 4,000 rpm for 30 min at 4 °C. The cell pellet was resuspended in complete media and freeze-thawed four times. The cell suspension was then centrifuged at 4,000 rpm for 20 min at 4 °C. The supernatant was collected and centrifuged in consecutive discontinuous CsCl gradients. After two rounds of density centrifugation, virions were collected, diluted in viral preservation media, and stored at −70 °C. The specific activity was 2×10^5 cpm/mg of labeled virus.

Preparation of Monoclonal Anti-fiber Antibody—Purified adenovirus type 5 fiber was prepared as described (gift of Einar Everitt) (16). The fiber protein was used to immunize BALB/c mice to generate monoclonal anti-fiber antibodies (mAbs) as previously described (17). The specificities of the mAbs were verified by Western blotting using purified wild type Ad5 as antigen. Selected positive hybridomas were cloned twice on soft agar and used to produce ascites fluid in BALB/c mice. Assays of end point cytopathic effects of adenovirus with purified antibody clone 7 (αfiberAb#7) showed that 1 μg of antibody completely neutralized 1×10^{10} particles of adenovirus.

Binding and Internalization of αfiberAb#7-coated ^{35}S-Labeled Virus—To measure binding of adenovirus to cell surfaces, monolayers of HeLa cells grown in 60-mm plates were incubated with ^{35}S-labeled adenovirus (8.31×10^{10} particles/plate) at 4 °C. The virus was preincubated (30 min at room temperature) with 83.1 μg of αfiberAb#7 or irrelevant mAb (anti-influenza neuraminidase antibody; PY203) prior to addition to cells. This amount of antibody represents a 10-fold excess of that amount required to completely neutralize adenoviral infection. The incubation allowed binding without cellular internalization of virions. After a 1-h incubation period, the cells were washed twice with ice-cold PBS. Virus adherent to the cell membrane was then harvested by treating plates with 1 ml of 0.1 N NaOH for 5 min at room temperature. Quantification of adherent virions was accomplished by addition of cell lysate to 4 ml of Scintiverse (Fisher Scientific) and analyzed by scintillation.

To measure adenovirus internalization, HeLa cells were incubated with antibody-coated ^{35}S-labeled adenoviruses, except the incubation was done at 37 °C. After incubation, cells were washed twice with PBS and treated with 0.1 N NaOH as above. The total amount of virus within the cells was determined as before.

Preparation of Molecular Conjugate-DNA Complexes—Human transferrin-poly(L-lysine) conjugate (hTfpL; Serva Biochemical) consists of human transferrin covalently linked to poly(L-lysine) with an average chain length of 300 lysine monomers. Conjugate-DNA complexes were formed by dilution of 6 μg of plasmid DNA in 350 μl of 150 mM NaCl, 20 mM HEPES, pH 7.3 (HBS) followed by addition of 8 μg of hTfpL diluted in 150 μl of HBS. Complexes were formed by incubation for 30 min at room temperature.

The hTfpL/AdpL conjugate contains both an adenovirus and a human transferrin domain. The ternary complexes (hTfpL/AdpL·DNA) were prepared by combining the epitope-tagged adenovirus P202-Ad5 (2.5×10^{10} particles) with poly(L-lysine)-conjugated monoclonal antibody (10) MP301pL (1.25 μg) diluted in 250 μl of HBS. The mixture was incubated at room temperature for 30 min. Plasmid DNA (6 μg) diluted in 125 μl of HBS was added to the mixture and incubated for an additional 30 min at room temperature. In order to completely condense the DNA and add a human transferrin domain to the complex, human transferrin-polylysine conjugates (6 μg) diluted in 125 μl of HBS were added to the complex and incubated for 30 min at room temperature. The reporter DNA used for experiments was pCMVL, a plasmid containing the *Photinus pyralis* luciferase gene under the control of the cytomegalovirus enhancer-promoter (18).

Effect of αfiberAb#7 on Facilitation of Molecular Conjugate-mediated Gene Transfer by Free Adenovirus—In order to determine the effect of αfiberAb#7 on facilitation of molecular conjugate-mediated gene transfer by free adenovirus, hTfpL·DNA complexes were prepared as described above. Adenovirus P202-Ad5 (2.5×10^{10} particles) was incubated with 25 μg of αfiberAb#7, an irrelevant mAb (PY203), or an equivalent volume of HBS for 30 min at room temperature prior to addition to the hTfpL·DNA complexes. The hTfpL·DNA complexes and antibody-treated adenoviruses were then added to 60-mm tissue culture plates containing HeLa cells (80% confluent) in 1 ml of low serum media that had previously been cooled to 4 °C for 15 min. The plates containing the complex/adenovirus/antibody mixture were returned to 4 °C and incubated for 1 h to allow for the complexes and free adenovirus to bind without internalizing. Immediately following incubation, the plates were washed twice with ice-cold low serum media to remove any unbound conjugates and free adenovirus. The plates were then incubated at 37 °C for 30 min following a 30-min gradual warm-up at room temperature. High serum media was then added to the cells prior to a 16-h incubation at 37 °C. After this incubation, cells were harvested for analysis of luciferase gene expression. Cell lysates were standardized for total protein content and analyzed for luciferase enzyme activity. Results were expressed as light units per 25 μg of total cellular protein.

Effect of αfiberAb#7 on Adenovirus-linked Molecular Conjugate-mediated Gene Transfer—In order to determine the effect of αfiberAb#7 on gene transfer by adenovirus-linked molecular conjugate complexes, ternary complexes containing adenovirus and human transferrin domains were prepared as described above. Adenovirus P202-Ad5 (2.5×10^{10} particles) was incubated with 25 μg of either αfiberAb#7 or PY203, or with an equal volume of HBS, for 30 min at room temperature prior to the formation of the hTfpL/AdpL complexes. The antibody-treated complexes were then added to 60-mm HeLa plates. The cells were incubated, washed, and harvested as above. Results were also expressed as light units per 25 μg of total cellular protein.

RESULTS

Effect of αfiberAb#7 on Binding and Internalization of Adenovirus—In order to determine the effect of αfiberAb#7 on binding and internalization of adenovirus, ^{35}S-labeled virions were incubated with αfiberAb#7 (10 μg of mAb/1.0×10^{10} particles) prior to delivery to HeLa cells. Virions labeled with [^{35}S]methionine were also incubated with an irrelevant mAb, an anti-influenza neuraminidase antibody (PY203), as a control. The results of these two assays are shown in Fig. 1. To determine the degree of ^{35}S-labeled virion attachment to cell surfaces, counts per minute (cpm) of radiation were analyzed. The binding study indicates that when virions were

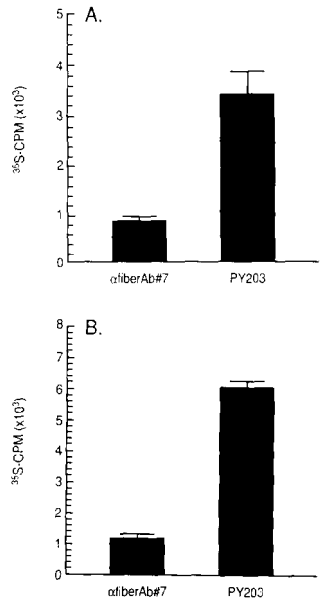

FIG. 1. **Effect of αfiberAb#7 on binding (*A*) and internalization (*B*) of adenovirus type 5.** ^{35}S-labeled adenovirus type 5 (2×10^5 cpm/mg) was preincubated with αfiberAb#7 or control mAb, PY203 (10 μg of mAb/1.0×10^{10} particles) for 30 min at room temperature prior to delivery to HeLa cells. This amount of antibody represents a 10-fold excess of that amount required to completely neutralize adenoviral infection. Experiments were performed in triplicate. In *A*, cells were incubated with antibody-coated virions for 60 min at 4 °C. This allowed for binding without internalization of virions. The cells were then washed with ice-cold PBS, and cells with adherent virus were harvested with 0.1 N NaOH. Cell lysates were then analyzed by scintillation. In *B*, cells were incubated with antibody-coated virions for 60 min at 37 °C, to allow for internalization of ^{35}S-labeled virions. Cell lysates were analyzed for viral uptake as above.

treated with αfiberAb#7, their ability to bind to HeLa cells was significantly decreased when compared to virions that had been preincubated with PY203. In the same manner, the internalization study indicates that the internalization of αfiberAb#7-treated virions significantly decreased when compared to virions treated with PY203. Thus, by blocking adenoviral binding, virions were also unable to accomplish cellular internalization. This established that the observed basis of antibody neutralization was blockade of adenoviral binding and internalization in target cells.

Effect of αfiberAb#7 on Facilitation of Molecular Conjugate-mediated Gene Transfer by Free Adenovirus—We have previously demonstrated that adenovirus can dramatically facilitate human transferrin-poly(L-lysine)-mediated gene transfer (9, 19). This derives from the fact that the adenovirus is co-internalized with the conjugate and, thus, adenovirus-mediated endosome disruption allows cell vesicle escape of the conjugate. The effect of αfiberAb#7-coated adenovirus on facilitation of molecular conjugate-mediated gene transfer in HeLa cells is shown in Fig. 2. When PY203 was preincubated with adenovirus and co-delivered with the hTfpL·DNA complex, gene transfer efficiency was unaltered when compared to complexes co-delivered with antibody-free virions. However, when αfiberAb#7-treated adenovirus and hTfpL·DNA complexes were co-delivered to cells, gene transfer was reduced by approximately 94%. Values for hTfpL + Ad and hTfpL + Ad + αfiberAb#7 are significantly different ($p < 0.0001$), while values for hTfpL + Ad and hTfpL + Ad + PY203 are statistically the same ($p > 0.05$). Thus, this confirms that adenoviral entry is a prerequisite for viral facilitation of molecular conjugate-mediated gene transfer.

Effect of αfiberAb#7 on Adenovirus-linked Molecular Conjugate-mediated Gene Transfer—Since the blocking antibody ablated the ability of free virus to facilitate molecular conjugate-mediated gene transfer, we wondered whether ablation of binding in the linked configuration would nevertheless allow retention of the endosome disruption capacity of the virus. Whereas one might expect a difference in gene transfer based on the loss of one entry mechanism, the fact that transferrin receptors are found in excess in HeLa cells makes this difference insignificant. The effect of αfiberAb#7 on adenovirus-linked molecular conjugate-mediated gene transfer is shown in Fig. 3. Gene transfer for linked-complexes that were preincubated with PY203 was as efficient as that observed for antibody-free linked-complexes. When linked complexes were then preincubated with αfiberAb#7, gene transfer was also as efficient as previously observed. Values for hTfpL/AdpL, hTfpL/AdpL + αfiberAb#7, and hTfpL/AdpL + PY203 are statistically the same ($p > 0.05$). Since the virions that were part of the complex lacked the ability to bind to their receptor, this result implied that hTfpL/AdpL·DNA complexes internalize only by an alternate non-adenoviral pathway. The binding-incompetent virions were nonetheless able to mediate vesicle disruption after internalization. In addition, adenovirus incubated with αfiberAb#7 at pH 6.0 prior to addition to cells was unable to efficiently enter cells via the receptor-mediated endocytosis pathway (data not shown). This result indicates that the monoclonal antibody remains bound to the virion at the pH of endosome vesicles and, thus, the fiber protein is not free to participate in endosome lysis. This result is consistent with the concept that adenoviral binding and vesicle disruption are not functionally linked. It is also consistent with our previous finding that hTfpL/AdpL·DNA complexes may function with high efficiency in gene transfer to erythroid cells, such as the K562 cell line, which have a very small number of adenovirus receptors (11).

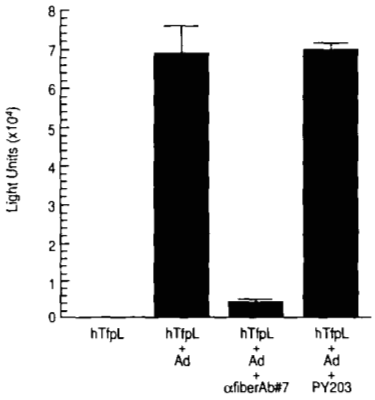

FIG. 2. **Effect of αfiberAb#7 on facilitation of molecular conjugate-mediated gene transfer by free adenovirus.** Adenovirus P202-Ad5, preincubated with αfiberAb#7 or PY203 (10 μg of mAb/1.0 × 10^{10} particles), and hTfpL·DNA complexes were co-delivered to HeLa cells. The cells were then incubated for 60 min at 4 °C to allow for binding without internalization of hTfpL·DNA and free virions, and washed to remove any unbound conjugate and adenovirus. This was followed by incubation at 37 °C to allow for virus and conjugate uptake. After 16 h, cell lysates were standardized for protein content and analyzed for luciferase activity. Results are expressed as light units per 25 μg of total cellular protein. Experiments were performed in triplicate.

DISCUSSION

Multi-functional molecular conjugates may be constructed that are capable of high efficiency gene transfer (10, 11). They possess both specific and efficient internalization and endosome lysis mechanisms. When the ligand domain of the conjugate is recognized by the appropriate cell surface receptor, the molecular conjugate-DNA complex is internalized by the receptor-mediated endocytosis pathway. To prevent endosome-internalized DNA from being trapped within the cell vesicle, we have incorporated adenovirus, which mediates endosome disruption, into the conjugate design. However, in doing so, the potential for cell specific targeting is undermined since in this configuration, the conjugate can bind by either the ligand domain or the adenovirus. Thus, we sought to ablate adenoviral binding by treating the conjugate with a neutralizing monoclonal anti-fiber antibody.

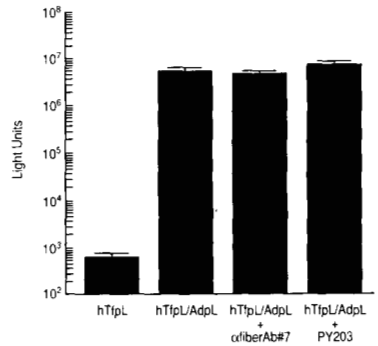

FIG. 3. **Effect of αfiberAb#7 on adenovirus-linked molecular conjugate-mediated gene transfer.** Adenovirus P202-Ad5 was preincubated with αfiberAb#7 or PY203 prior to formation of the hTfpL/AdpL·DNA complexes (10 μg of mAb/1.0 × 10^{10} particles). The hTfpL/AdpL·DNA complexes were then delivered to HeLa cells and incubated for 60 min at 4 °C to allow for binding of the complexes. Cells were washed and harvested as described in Fig. 2. Results are reported as light units per 25 μg of total protein. Experiments were performed in quadruplicate.

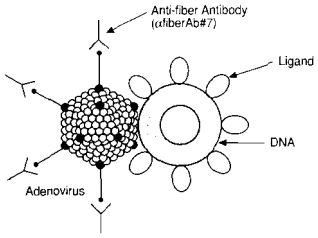

FIG. 4. **Multi-functional molecular conjugate vector that exploits adenovirus-mediated endosome lysis in a selective manner.** Adenovirus is rendered binding-incompetent by interaction with a monoclonal anti-fiber antibody. The complex will thus enter cells by non-adenoviral pathways with virus serving exclusively as an endosome lysis agent.

We wondered whether blockade of adenoviral binding would impede the endosome lysis activity of the virus. In this regard, we considered what is known of the adenoviral entry pathway. At pH 7, the adenovirus fiber first binds to an uncharacterized receptor on the cell surface. The virion is then engulfed by a clathrin-coated pit and internalized into an endosome. After internalization, acidification of the endosome results in endosome disruption, allowing the virion to proceed to the nucleus to complete its life cycle (13). The exact mechanism of endosome disruption has not been completely delineated. However, monoclonal antibodies against the penton protein have been shown to selectively block endosome lysis (15). Thus, the penton base and/or peripentonal capsid components appear to be the crucial capsid proteins involved in endosome disruption. Based on this, we hypothesized that we could block adenoviral binding with an anti-fiber antibody without disrupting endosome lysis activity.

The results reported here show that it is possible to construct multi-functional molecular conjugates that are able to mediate high efficiency gene transfer without having the adenovirus act as a competing ligand by using neutralizing amounts of an anti-fiber antibody. This is depicted in Fig. 4. While this maneuver can eliminate adenoviral binding, other possible sources of nonspecific binding may arise from the conjugate's design. For instance, it is known that polylysine can bind nonspecifically to certain cell lines (20). However, specific maneuvers may also be employed to address this nonspecificity where relevant (21). In this way, the specificity of the conjugate ligand domain may be preserved. We have also shown that it is possible to preserve endosome lysis activity in the absence of adenoviral binding. These findings demonstrate that in the process of adenoviral entry, binding and endosome disruption are not functionally linked.

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