Secreted and Transmembrane Mucins Inhibit Gene Transfer with AAV4 More Efficiently than AAV5*

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Robert W. Walters §§, Joseph M. Pilewskï, John A. Chiorini † † †, and Joseph Zabner ‡‡

From the Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, the Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and the NIDCR, National Institutes of Health, Bethesda, Maryland 20892

Adeno-associated virus (AAV) is a promising vector for gene transfer in cystic fibrosis. AAV4 and AAV5 both bind to the apical surface of differentiated human airway epithelia, but only AAV5 infects. Both AAV4 and AAV5 require 2,3-linked sialic acid for binding. However, AAV5 interacts with sialic acid on N-linked carbohydrates, whereas AAV4 interacts with sialic acid on O-linked carbohydrates. Because mucin is decorated with O-linked carbohydrates, we hypothesized that mucin binds AAV4 and inhibits gene transfer. To evaluate the effect of secreted mucin, we studied mucin binding and gene transfer to COS cells and the basolateral membrane of well differentiated human airway epithelia. AAV4 bound mucin more efficiently than AAV5, and mucin inhibited gene transfer with AAV4. Moreover, O-glycosidase-pretreated mucin did not block gene transfer with AAV4. Similar to secreted mucin, the transmembrane mucin MUC1 inhibited gene transfer with AAV4 but not AAV5. MUC1 inhibited AAV4 by blocking internalization of the virus. Thus, O-linked carbohydrates of mucin are potent inhibitors of AAV4. Furthermore, whereas mucin plays an important role in innate host defense, its activity is specific; some vectors or pathogens are more resistant to its effects.

Previous work implicates mucin as a key component of the innate host defense system against pathogens (1–5). In particular, mucin acts as a general physical barrier that when coupled with ciliary action rapidly clears inhaled particles out of the lung (4). In addition, mucin might act as a specific defense. That is, the molecular components of mucin could potentially act as a soluble receptor system. This system might be important in the removal of specific pathogens (e.g. viruses that interact with carbohydrate structures in mucin). Mucin binds both bacteria (e.g. Hemophilus influenzae and Moraxella catarrhalis) and viruses (e.g. rotavirus and influenza) often via sialic acid (3, 6–9). Interestingly, several of these organisms use sialic acid as a cellular receptor (6, 10–13). This raises the question of what controls the balance between pathogen binding to cellular receptors leading to infection and binding to mucin leading to clearance. More specifically, could the binding specificity of the pathogen control this balance? We took advantage of our recent findings with adeno-associated viruses to address this question.

Recombinant adeno-associated viruses (AAV) hold promise for gene transfer to several tissues including the airway (14–20). AAV2 was the first primate AAV to be cloned and has been studied extensively (21). This vector was recently shown to mediate Factor IX gene transfer to the muscle of humans, making hemophilia one of the first human genetic diseases to be partially corrected by gene transfer (20). AAV2 has also been investigated for gene transfer of the cystic fibrosis transmembrane conductance regulator cDNA to airway epithelia in cystic fibrosis in vitro and in vivo (15, 16, 22–25). However, because AAV2 is inefficient, other serotypes have been investigated for targeting the apical surface of airway epithelia.

Both AAV4 and AAV5 bind the apical surface of human airway epithelium more efficiently than AAV2; however, only AAV5 infects (26, 27). Hence, why does AAV4 bind but not infect? Surprisingly, both AAV4 and AAV5 require 2,3-linked sialic acid for binding and infection (28, 29). However, AAV4 requires sialic acid present in O-linked oligosaccharides, whereas AAV5 requires sialic acid in N-linked oligosaccharides. Because mucin contains primarily O-linked oligosaccharides, we tested the hypothesis that mucin binds and inhibits gene transfer with AAV4, not AAV5.

MATERIALS AND METHODS

Cells and Culture—COS-7 cells were cultured on 96-well plates (Corning Costar, Corning, NY) in Eagle's minimal essential media (EMEM, Sigma) supplemented with 10% fetal calf serum (Sigma), 1% nonessential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Airway epithelial cells obtained from trachea and bronchi of lungs removed for organ donation were isolated by enzyme digestion and seeded at 5 × 10⁵ cells/cm² onto collagen-coated 0.6-cm² area Millicell polycarbonate filters (Millipore) (30, 31). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and air. 24 h after plating, mucosal medium was removed, and cells were allowed to grow at the air-liquid interface (30–32). Culture media consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium, 5% Ultraserase G (Bispeira SA, Cergy-Saint-Christophe, France), 100 units/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 0.12 unit/ml insulphin. Epithelia were allowed to reach confluence and develop a transepithelial electrical resistance and a ciliated apical surface (31, 33).

Adeno-associated Viruses—Recombinant AAV4 and AAV5 were produced as described previously (34). AAV4/β-galactosidase and AAV5/β-galactosidase were prepared by triple plasmid cotransfection of COS cells.

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† † † To whom correspondence may be addressed: NIH 10/JN113, 10 Center Dr., MSC 1190, Bethesda, MD 20892. Tel.: 301-496-4279; Fax: 301-402-1228; E-mail: jchiorini@dir.nidcr.nih.gov.

‡‡ To whom correspondence may be addressed: University of Iowa College of Medicine, 500 EMBR, Iowa City, IA 52242. Tel.: 319-353-5511; Fax: 319-353-7623; E-mail: joseph-zabner@uiowa.edu.

The abbreviations used are: AAV, adeno-associated virus; EMEM, Eagle's minimal essential media; MUC29, truncated MUC1 containing only 2 repeats.

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cells using calcium phosphate cotransfection (Invitrogen). Cells were harvested and pelleted 72 h post-transfection, resuspended in tissue dissociation buffer (140 mM NaCl, 5 mM KCl, 0.7 mM K2HPO4, 25 mM Tris/HCl, pH 7.4), and stored at -70 °C. Samples were thawed at 37 °C, treated with benzonase (Sigma) at 20 units/ml and sodium deoxycholate at 0.5% for 1 h, and homogenized (20 strokes in a Wheaton B homogenizer). CsCl was added to 1.4 g/cm3, and the homogenate was centrifuged (SW 40 rotor at 38,000 rpm) for 65 h at 20 °C. Gradient fractions with a refractive index of 1.371–1.373 were pooled. Viruses were titered by Southern blot and transmission electron microscopy. Titers ranged between 1 × 1011 and 1 × 1012 particles/ml. The ratio of infectious units to particles is the same for AAV4 and AAV5 in COS cells (35, 36).

**Mucin Binding Assay**—The binding of AAV4/β-galactosidase and AAV5/β-galactosidase to mucin was measured using a dot blot assay. Bovine submandibular gland mucin (Sigma) contains a major glycoprotein (82% glycosylated) and a minor glycoprotein (65% glycosylated). Both proteins are glycosylated with O-linked carbohydrate only, and nearly all sites contain sialic acid (37). Mucin was adsorbed onto 96-well enzyme-linked immunosorbent assay plates (Corning Costar) for 1 h at 37 °C in 5-fold dilutions starting from 1 mg/ml. Plates were washed three times with phosphate-buffered saline and then blocked overnight with 5% bovine serum albumin at 4 °C. AAV4 and AAV5 were added at 1 × 1010 particles/ml in EMEM for 2 h at 37 °C. Plates were rinsed three times with EMEM, and bound virus was quantified. Samples were treated with trypsin-EDTA for 2 h at 37 °C, subjected to three freeze/thaw cycles, and blotted onto a nylon membrane (Ambion). AAV viral DNA was detected by hybridizing with a 32P-labeled β-galactosidase cDNA probe. Unhybridized probe was washed twice with 2 × SSC and 0.1% SDS at 20 °C for 15 min, once with 0.5 × SSC and 0.1% SDS at 55 °C for 1 h, and once with 0.5 × SSC and 0.1% SDS at 65 °C for 30 min. Dot blots were developed and quantitated using a PhosphorImager (Molecular Dynamics) (26, 38).

**Mucin Competition and MUC1 Expression**—Mucin competition was carried out by pretreating virus with mucin for 30 min at 37 °C. Virus alone or virus plus mucin was added to cells in equal volumes of EMEM for 1 h at 37 °C. Cells were rinsed twice with EMEM and incubated at 37 °C. In COS cells, 2000 particles/cell were pretreated with mucin (concentrations up to 1 mg/ml), and cells were assayed for gene transfer 3 days later. In cells expressing MUC1 (see below), virus was treated with 0.01 mg/ml mucin.

**FIG. 1.** Binding of AAV4 and AAV5 to mucin. A, dot blot of AAV4 and AAV5 binding to mucin. Mucin concentration increases in 5-fold increments from left to right. B, quantitation of mucin binding with AAV4 (○) and AAV5 (●). Data are the mean binding ± S.E. (n = 3).

**FIG. 2.** Effect of mucin on AAV4- and AAV5-mediated gene transfer to COS cells. A, dose response of mucin competition with AAV4 (○) and AAV5 (●). B, competition of AAV4 with mucin versus mucin lacking O-linked carbohydrate. Data are the mean gene transfer ± S.E. (n = 3–9). LU, light units; asterisk, p < 0.05.

**FIG. 3.** Effect of mucin on AAV4- and AAV5-mediated gene transfer to airway epithelia. A, scanning electron micrograph of well differentiated human airway epithelia indicating mucus (M), cilia (C), columnar epithelial cells (E), and basal cells (B) growing on a filter support (F). B, gene transfer with AAV4 and AAV5 from the basolateral membrane of human airway epithelia. Data are the mean gene transfer ± S.E. (n = 6). LU, light units; asterisk, p < 0.05.
were pretreated with 0.1 mg/ml mucin and added to the basolateral membrane of epithelia. Epithelia were assayed 2 weeks later.

O-Linked carbohydrate was removed from mucin by treating a 2 mg/ml solution with 0.1 unit/ml O-glycosidase at pH 5.0 for 24 h at 37 °C. Deglycosylated mucin was purified with column chromatography using boronic acid conjugated to Sepharose beads (Pierce). The recovery was determined by absorbance at 250 nm, and equal concentrations of protein were used to test competition of glycosylated mucin versus deglycosylated mucin.

Human MUC1 containing 22 tandem repeats or truncated MUC1 containing only 2 repeats (MUC1Δ29) in pDNA3 was expressed in COS cells using a LipofectAMINE transfection as directed (Invitrogen). MUC1 expression and AAV gene transfer were studied 24 h later.

**Electron Microscopy and Immunocytochemistry**—Mucus in airway epithelial cultures was evaluated by scanning electron microscopy. Traditional aqueous steps were avoided (39, 40). Cell cultures were gently immersed in 1% osmium tetroxide dissolved in 3 M perfluorocarbon (PFC-Fluorinert FC87) for 2 h and rinsed with perfluorocarbon. Samples were placed in 50:50 ethanol:hexamethyldisilazane, washed twice in 100% hexamethyldisilazane, and air-dried. Filters were sputter-coated with gold/palladium and imaged in an Hitachi S-4000.

MUC1 expression was evaluated by immunocytochemistry. Cells were rinsed with phosphate-buffered saline and then fixed with 4% paraformaldehyde for 20 min at 20 °C. Unless otherwise noted, SuperBlock (Pierce) was used to wash and dilute reagents. Cells were washed twice for 10 min, and mouse anti-MUC1 monoclonal antibody (1:50, Serotec, Raleigh, NC) was added for 1 h at 37 °C, rinsed twice for 10 min, and incubated with donkey α-mouse IgG conjugated with fluorescein isothiocyanate fluorophore (1:500, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at 37 °C. Cells were rinsed twice with phosphate-buffered saline for 10 min and mounted using Vectorshield containing 4,6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA). Cell staining was evaluated by indirect immunofluorescence microscopy.

**Gene Transfer Assays**—Gene transfer was measured as described previously (26). We measured β-galactosidase activity using a commercially available method (Galacto-Light, Tropix Inc., Bedford, MA). After rinsing with phosphate-buffered saline, cells were lysed by incubation with 120 μl of buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminoxyacyclohexane-N,N,N′,N″-tetraacetic acid, 10% glycerol, and 1% Triton X-100) for 15 min. Light emission was quantified in a luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**Cell Binding and Internalization Assays**—The binding and internalization of AAV4/β-galactosidase to COS cells were measured using a dot blot assay (26, 38). Experiments were carried out by binding AAV4 at 2 × 10^9 particles/ml for 60 min at 4 °C. To remove unbound virus, cells were rinsed three times with EMEM and either harvested to determine binding or incubated at 37 °C to allow internalization. Following 15-min or 3-h incubations, cells were treated with trypsin-EDTA for 30 min at 20 °C to remove AAV not internalized. Cells were pelleted, rinsed three times with EMEM, and then harvested. Samples were subjected to three freeze/thaw cycles, blotted, and probed as described above.

**Data Analysis**—Statistical significance was determined with a Student's t test. The values of p < 0.05 were considered significant. Competition binding curves with mucin were analyzed to obtain the approximate IC_{50} for inhibition. The curves were fit as a two-site competition using nonlinear regression (Prism, GraphPad software).

**RESULTS**

**AAV4 Binds Mucin More Efficiently than AAV5**—Using a dot blot assay, we studied in vitro binding of AAV4 and AAV5 to purified mucin that contains O-linked carbohydrate only (37). Starting with equal amounts of AAV4 and AAV5, we observed significant binding with AAV4, which was dependent on the dose of mucin (Fig. 1A). In contrast, AAV5 bound poorly even with large amounts of mucin present. To further understand these interactions, we quantified binding (Fig. 1B). An analysis
of AAV4 revealed a saturating binding curve, suggesting that the interaction between AAV4 and mucin is specific. However, with AAV5, the binding seemed to increase in direct proportion to mucin, suggesting that the interaction with AAV5 and mucin is primarily nonspecific. Hence, mucin binds AAV4 more efficiently than AAV5.

**Soluble Mucin Inhibits Gene Transfer with AAV4 Greater than AAV5**—To determine whether mucin inhibits AAV4 or AAV5, we studied gene transfer to COS cells with these viruses. AAV4 and AAV5 infected COS cells with similar levels of gene transfer (Fig. 2A). However, increasing amounts of mucin inhibited AAV4 significantly more than AAV5. More to the point, mucin was a more “effective” inhibitor of AAV4 than AAV5. The maximum inhibition resulted in a 10-fold decrease in gene transfer with AAV4 as compared with a 2-fold decrease with AAV5. In addition, mucin was a more “potent” inhibitor of AAV4 than AAV5. The concentration required to half-maximally inhibit AAV4 was ~50 times lower than the concentration required to half-maximally inhibit AAV5. Hence, mucin inhibits infection with AAV4 more efficiently than AAV5.

Because AAV4 interacts with O-linked carbohydrates, we predicted that the O-linked carbohydrates of mucin are required to inhibit AAV4. The exact nature of the carbohydrate is not known; hence, we tested this prediction by removing the O-linked carbohydrate from mucin and studied its ability to block infection with AAV4. At 0.01 mg/ml mucin, deglycosylated mucin no longer inhibited AAV4 infection (Fig. 2B). Thus, O-linked carbohydrates in mucin are directly responsible for this inhibition.

**Mucin Inhibits AAV4 Infection of Human Airway Epithelia but Not AAV5**—To study the effect of mucin on gene transfer to airway epithelia, we took advantage of the fact that mucin is selectively expressed and secreted through the apical membrane (Fig. 3A) (4). Therefore, we studied the ability of AAV4 and AAV5 to infect these differentiated cultures from the basolateral surface. In contrast to apical infection, both AAV4 and AAV5 infected airway epithelia from the basolateral surface and with similar efficiency (Fig. 3B) (26). Therefore, some factor at the apical surface blocks AAV4 but not AAV5. If this factor is mucin, then the addition of mucin to the basolateral surface should also limit basolateral infection with AAV4. The infection in the presence of 0.1 mg/ml mucin blocked basolateral gene transfer with AAV4 but not AAV5. Hence, the polarity of mucin correlates with the polarity of infection with AAV4, suggesting that mucin inhibits apical infection with AAV4.

**Transmembrane Mucins Inhibit AAV4 but Not AAV5 Infection**—In addition to secreted mucins, airway epithelia also present transmembrane mucins on the apical membrane (41). To test the hypothesis that transmembrane mucins also inhibit infection with AAV4 and not AAV5, we expressed MUC1 (containing either 22 tandem repeats or 2 tandem repeats) in COS cells and studied gene transfer with these viruses. Approximately 70% of cells were transfected (data not shown). We confirmed MUC1 expression by immunocytochemistry. MUC1 is present on the surface of cells expressing MUC1 with 22 tandem repeats but not on the surface of control cells (Fig. 4, A and B). The expression of MUC1 containing 22 tandem repeats significantly decreased infection with AAV4 (Fig. 4C). MUC1Δ29 showed a trend toward decreased infection with AAV4, but this difference was not statistically significant. In contrast to AAV4, AAV5 infection was not inhibited by recombinant MUC1 expression. Hence, the transmembrane mucin MUC1 inhibits only AAV4, and the effect is dependent upon the number of mucin tandem repeats.

These observations raise the question as to whether MUC1 and soluble mucin together have synergistic or additive effects. Thus, we studied AAV4-mediated gene transfer to truncated MUC1-expressing cells in the presence of submaximal concentrations of soluble mucin (0.01 mg/ml). Together, soluble mucin and MUC1Δ29 inhibited gene transfer greater than either mucin alone, and this effect was additive (Fig. 4D). Moreover, soluble mucin reconstitution inhibition with the truncated MUC1, further supporting its role as a soluble receptor system.

**Transmembrane Mucin Inhibits Internalization of AAV4**—To understand how MUC1 inhibits gene transfer with AAV4, we studied the binding and entry of AAV4 in MUC1-expressing cells. The total binding of AAV4 was not affected by MUC1 expression (Fig. 5, A and B). However, the cells expressing full-length MUC1 internalized less AAV4 after a 15-min pulse than the controls or cells expressing truncated MUC1, and this inhibition was more obvious after 3 h (Fig. 5C). Hence, MUC1 blocks AAV4 by inhibiting internalization, and the ef-
fect is dependent upon the number of mucin tandem repeats. Interestingly, human MUC1 typically has between 47 and 80 tandem repeats. This finding plus the fact that not every cell expressed MUC1 suggests that our results may underestimate the impact of transmembrane mucins on gene transfer with AAV4.

**DISCUSSION**

Secreted and transmembrane mucins inhibit gene transfer with AAV4 more effectively and potently than AAV5. Therefore, AAV5 infects airway epithelia from the apical membrane in the presence of endogenous mucin/mucus (26). Because both AAV4 and AAV5 require 2,3-linked sialic acid to infect cells but differ with respect to other aspects of the carbohydrate (i.e. O-linked versus N-linked), we conclude mucin has a specific binding function. This finding is relevant both for the development of airway and intestine-targeted vectors and for understanding innate host defenses against invading microorganisms.

**Implications for Gene Transfer—Rational development of gene transfer vectors requires an understanding of the steps required and barriers that prevent efficient delivery of genes.** Recent work has focused on elucidating the barriers that prevent gene transfer from the apical membrane of airway epithelia. For instance, AAV5 is better than AAV2, because there are more apical receptors for AAV5. However, other barriers that precede receptor binding may also be important. For example, mucin seems to have activity against several candidate gene transfer vectors including adenovirus (41). In addition, previous work showed that mucin can be used to purify AAV5 (42). Given that AAV5 binds N-linked carbohydrates, these two points raise the question of whether AAV5 binds mucin specifically and whether mucin affects AAV5 function in vitro. We observed that mucin inhibits AAV4 greater than AAV5, and AAV5 infects differentiated airway epithelia even in the presence of endogenous mucin/mucus both in vitro and in vivo as described above and previously (26). Hence, mucin specifically blocks vectors that interact with O-linked carbohydrates such as AAV4, and its impact on other vectors (i.e. AAV5) may not be therapeutically significant. These observations should aid in our understanding, development, and use of gene transfer vectors.

**Implications for Innate Host Defense and Viral Pathogenesis—** Whereas mucin clearly plays a role in innate host defense, we do not understand the exact nature of its role. Mucin can inhibit pathogens that interact with O-linked carbohydrate (one example is rotavirus (13)). To circumvent this barrier, rotavirus evolved specific enzymatic activity that allowed the virus to digest its way through the mucin layer (43). In addition to this complex mechanism, our work suggests a different and perhaps simpler evolutionary mechanism for evading the mucin barrier. More specifically, recognizing N-linked carbohydrate as opposed to O-linked carbohydrate seems a significant step forward with respect to the ability of AAV to infect airway epithelia. In addition to its importance for this gene transfer vector, we speculate that this mechanism generally may be more applicable to pathogen evolution, possibly accounting for variability in virulence of viruses such as influenza.

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