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Chimeric Ad5 Vectors Expressing the Short Fiber of Ad41 Show Reduced Affinity for Human Intestinal Epithelium

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Abstract: Altering adenovirus tropism has attracted increased attention in recent years to improve gene delivery. We constructed a recombinant Ad5 vector carrying the non-CAR (coxsackievirus and adenovirus receptor) binding short fiber of enterotropic Ad41 (Ad5SHORT) and tested its transduction efficiency on enterocytes. Ad5SHORT was engineered, in high titers similar to the parent vector, by homologous recombination in *Escherichia coli* BJ5183 (*recBC sbcBC*) and propagated on C7 cells. Western blotting confirmed the presence of Ad41 short fiber on Ad5SHORT while lack of CAR-binding was evident by the low transduction of CHO-CAR cells. Transduction efficiency of enterocytes, the natural target tissue for the fiber-"donor" virus Ad41, was tested in human intestinal biopsy cultures and in Caco-2 cells, including ulcerative colitis tissue and mucosal wound healing models. Ad5SHORT exhibited up to 23-fold lower transduction levels compared to Ad5 in human intestinal biopsy cultures and up to 13-fold in the in vitro systems. The differences with the in vitro systems were more pronounced when less differentiated cells were used. These studies highlight the potential for using this chimeric Ad5/Ad41 vector as a scaffold for the development of retargeted adenoviral vectors. Finally, our results suggest that the short fiber does not appear to be mediating, at least by itself, the increased enterocyte affinity of Ad41.

Keywords: Adenovirus; enterocytes; transduction; cell culture; explants

Introduction

Adenoviruses are extensively used as vectors for both gene delivery and research purposes. They offer several advantages such as the ability to transduce both dividing and nondividing cells, ease of production and purification in high titers, in vivo stability, relative safety concerning pathogenicity, and lack of germ line integration.[1,2] However, one major disadvantage is the lack of specificity toward the targeted cell populations, as well as reduced specificity for several tissues of interest. Adenoviruses enter cells mainly through receptor-mediated endocytosis.[3] The major entry pathway involves

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the attachment of the fiber "knob" of the virus to the coxsackievirus and adenovirus (CAR) receptor,[4,5] followed by internalization of the virus through clathrin-mediated endocytosis that is mediated by binding to anb3 and anb5 integrins on the cell surface.[6] Recently, however, heparan sulfate proteoglycans (HSGs), a family of plasma membrane proteins, have emerged as binding sites for adenoviruses[7,8] and appear to play an important role in liver transduction.[9,10]

Altering adenovirus tropism has attracted increased attention in the recent years. The goal is to increase gene transfer while improving specificity of virus binding to the tissue of interest. Decreased gene transfer especially is a major limitation for gene delivery to epithelial tissues, including gastrointestinal epithelium. The retargeting strategies fall into two general categories:[11] two-component systems where bispecific conjugates, typically an antibody against a component of the viral capsid and a targeting antibody or ligand, are used, and genetically modified vectors. In the latter case, two possibilities exist: genetic manipulation of capsid proteins, typically the fiber or the fiber knob, or replacement of the native capsid proteins by proteins from other adenoviral serotypes. Ideally, retargeted vectors will maintain little of their initial tropism, thus increasing selectivity for the new target tissue. Pseudotyped vectors can also prove useful tools in studying virus-receptor interactions, promoting our understanding of differences in adenoviral infection in different cell lines.

Ad41 belongs to the subgroup F of adenoviruses, along with Ad40. Both are enteric adenoviruses, responsible for nearly 20% of all cases of infant diarrhea. Ad41 exhibits several structural differences compared to the most commonly used Ad2 and Ad5. The most notable of these is that Ad41 particles carry two different fibers of different lengths and different primary sequences.[12] The long fiber is about 340 Å long and contains 562 amino acids (60.6 kDa). The short fiber is about 200 Å long and contains 387 amino acids (41.4 kDa). The two fibers are present in equal amounts on the virion.[13] Unlike the long fiber, the short fiber does not bind to CAR.[14] It was recently suggested that the short fiber further lacks ability to bind heparan sulfate.[15] The functions of the short fiber and its cellular receptor remain unknown. Furthermore, Ad41 virus lacks an RGD motif on the penton base, thus being unable to bind to avb3 and avb5 integrins on the cell surface.[16]

Due to the lack of CAR binding, Ad41 short fiber appears as a good candidate to be incorporated in retargeted vectors that could be further manipulated for targeting via bispecific antibodies. As receptors to Ad5 vectors are expected to be scarce on the luminal side of intestinal epithelia, targeting of the vector to alternate receptors may increase affinity. However, since the function of the short Ad41 fiber remains unclear, the possibility that it confers enterotropic nature by interacting with a, yet unknown, receptor in the gastrointestinal tract cannot be ruled out. A recombinant Ad5 adenoviral vector was engineered to express the short fiber of Ad41, by inserting the short fiber gene (1.2 kb) in the place of the L5 Ad5 gene under the control of the endogenous Ad5 fiber gene promoter (MLP). The recombinant vector was characterized by Western blotting and in transduction experiments with CAR-expressing cells in vitro. The ability of the vector to transfect intestinal epithelial cells was examined in human intestinal explants and in Caco-2 cell cultures. Furthermore, the vector was tested in explants from ulcerative colitis (UC) patients and in vitro models of restituting intestinal epithelium, since inflammatory bowel disease (IBD) tissue represents a promising target for gene delivery purposes.[17,18]

Experimental Section

Human Subjects. These studies were approved by the Institutional Review Board of the University of Michigan Medical Center (IRBMED No. 1998−0016). All subjects gave informed consent. Subjects were selected from among

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patients scheduled to undergo endoscopy for clinical indications. Selection was based on appropriate diagnosis (ulcerative colitis, Crohn's disease, or with endoscopically normal mucosa and no history of inflammatory bowel disease). Exclusion criteria were as follows: age less than 18 years or greater than 65 years, known HIV or viral hepatitis, coagulopathy, use of anticoagulants such as aspirin or coumarin, or inability to give informed consent. Two to six biopsies were obtained from each subject.

Materials. Tissue culture reagents were obtained from Invitrogen Co. (Carlsbad, CA), and tissue culture materials were from Becton Dickinson (Franklin Lakes, NJ) and Corning Inc. Life Sciences (Acton, MA). All other chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Adenoviral Vectors. Adenoviral type 5 (Ad5) vectors (Vector Core Laboratory, University of Michigan) were used for all experiments. Ad5CMV-luc vectors contained the luciferase reporter gene in the E1A/E1B region of the viral genome under the control of a CMV promoter.[19] Ad5CMV-betagal contained the nuclear targeted β-galactosidase (LacZ) gene.

Recombinant Ad5/Ad41 Vector Construction. Ad5SHORT-betagal and Ad5SHORT-luc are E1-deleted Ad5-based vectors that carry either the β-galactosidase (lacZ) or luciferase (luc) gene in the E1 region under control of the cytomegalovirus promoter (CMV). The short fiber gene from Ad41 was inserted in the chimeric vectors by homologous recombination in *Escherichia coli* BJ5183 (*recBC sbcBC*). The Ad41 short fiber gene was provided by Dr. Jay K. Kolls (Louisiana State University Health Sciences Center, New Orleans, LA). To isolate a short fiber-containing fragment for recombination, a shuttle vector containing the Ad41 short fiber gene was constructed using a pGEM-T Easy vector system (Promega, Madison, WI). Sequence upstream of the Ad5 fiber gene was synthesized by PCR (primers 5′ AACCGGAATTCTTTAATTATGAAATTTACTGTGACTTTTCTGC and 5′ ATCCGATTTAAATCTGCAACAACATGAAGATAGTGG) from the Ad5 pTG3602 plasmid with a unique *Swa*I site included in the 3′ primer as an insertion site for the Ad41 short fiber gene. The 5′ Ad5 fiber PCR fragment was ligated into the pGEM-T Easy vector at the PCR insertion site. The short fiber gene was isolated as a 1.1 kb *Bgl*II/*Bam*HI fragment from pVL1392 and cloned into the *Swa*I site at the 3′ end of the upstream Ad5 fiber gene sequence. A 5.6 kb *Hpa*I/*Pac*I restriction fragment from pTG3602 was cloned into the *Nde*I site in pGEM-T Easy to make pGEMrecS. The 3.3 kb *Hpa*I/*Pac*I DNA fragment included 783 bp of 3′ Ad5 fiber gene sequence and additional Ad5 sequence to the *Pac*I site at the right end of pTG3602. A 3.3 kb *Ngo*MIV fragment was isolated from pGEMrecS as the fragment for recombination with *Swa*I linearized pTG3602. Cotransfection produced the recombinant vector pTGrecS. PTGrecS was prepared in *E. coli* SCS110 (*dam dcm*) (Strategene, La Jolla, CA). β-Gal or luc genes were recombined into the *Cla*I site in the E1 region of pTGrecS and prepared in *E. coli*.[20] The large Ad plasmids were isolated using the Concert Maxiprep System (Gibco, Grand Island, NY). All vectors were verified by restriction mapping and sequencing of the fiber and reporter gene regions.

Virus Propagation and Titer. pTGrecS was treated with *Pac*I to release the Ad5SHORT genome. The Ad5SHORT DNA was transfected into C7 cells expressing Ad5 polymerase and preterminal protein to maximize titer.[21] Transfection, purification, and titering were performed according to established protocols[19] in the Vector Core Laboratory, University of Michigan. Stock vector was stored in aliquots at −80 °C in buffer containing 10% glycerol. The concentration of virus particles was determined by measuring optical density at 260 nm. The viral titer was determined by plaque assay in HEK 293 cells.

Western Analysis of Recombinant Virus Fiber. Purified virus was heated at 95 °C for 15 min in Laemmli buffer and subjected to 4−15% SDS−PAGE. Denatured and separated proteins were transferred to PVDF (Bio-Rad, Hercules, CA), and unstained protein markers were stained with Ponceau S (Sigma, St. Louis, MO) and destained in TBS (20 mM Tris, 137 mM NaCl, pH 7.6). The viral proteins bound to the PVDF membrane were blocked overnight at room temperature in 5% dry milk dissolved in TBS−0.1% Tween 20. A cross-reacting polyclonal antibody to Ad2, R72 (a kind gift of Dr. Marshall Horwitz, Albert Einstein College of Medicine, New York, NY), was added to the membrane at a dilution of 1:10 000. Peroxidase-conjugated donkey anti-rabbit antibody was applied as the secondary antibody (1:5000), and all steps for binding and detection were performed according to the manufacturer's instructions that accompanied the chemiluminescent reagents used for detection (ECL Plus, Amersham, Piscataway, NJ).

Adenoviral Transduction Studies in CHO Cells. CHO-K1 cells (ATCC, CCL-61) were grown in Ham's F12 medium containing 2mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum (FBS). *dhfr*$^-$ CHO cells transfected with human CAR cDNA plasmid or pcDNA control plasmid encoding dihydrofolate reductase (CHO-CAR or CHO-pcDNA) were grown in alpha-minus MEM and 10% dialyzed FBS for selection of the plasmid (a kind gift of Dr. Jeffrey M. Bergelson, Children's Hospital of Philadelphia, Philadelphia, PA). Transduction assays of CHO-K1, CHO-CAR, or CHO-pcDNA were performed on 10^5 cells. Ad5CMV-betagal and Ad5SHORT-betagal were applied at 500, 5000, or 50 000 PFU (plaque-forming units) (corresponding MOIs of ∼15 000, ∼150 000, and ∼1 500 000,

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respectively). Virus was applied to cells for 60 min, the cells were washed, and the cells were incubated for 48 h. β-Galactosidase expression was visualized with X-gal staining.

Isolation and Transduction of ex Vivo Intestinal Organ Cultures. Human intestinal biopsies from normal and diseased (IBD) patients were obtained at the time of endoscopy at University of Michigan Hospital. Six biopsies were obtained from each patient following informed consent. Biopsies were collected in 10 mL of transport medium [80% CMRL medium 1066 + 20% F-12 (Ham) nutrient mixture] that had been equilibrated with 95% O_2/5% CO_2 and cooled on ice. Explants were weighed, but not rinsed, and placed luminal side up on a 310 μm pore size screen (Cole Parmer Instrument Company, Vernon Hills, IL) cut to fit across the inner-well shoulders of organ culture dishes (Falcon, Oxnard, CA). Each well contained 2.15 mL of explant culture medium [80% CMRL medium 1066 + 20% F-12 (Ham) nutrient mixture, FBS 10%; glucose 10 mM; GlutaMax I 2 mM; sodium selenite 0.1 μM; zinc sulfate 3 μM; menadione sodium bisulfite 145 nM; vitamin E 45 nM; sodium penicillin G 100 units/mL; and gentamicin 50 μg/mL]. Medium was spontaneuously drawn over the explant, which was not fully submerged. All cultures were kept on ice prior to virus addition. Application of 1×10^9 viral particles in a volume of 4 μL to the apical side of the explant was performed at room temperature. All steps from biopsy procurement to addition of virus occurred in 45−60 min. Following virus application the organ culture dishes were placed in a polysterene modular incubator chamber (Billups Rothenberg Incorporated, Del Mar, CA) and gassed with approximately 55 L of filtered 95% O_2/5% CO_2 over 15 min as measured by a Barnant/Gilmont flowmeter (Fisher Scientific, Pittsburgh, PA). The chamber was placed at 37 °C for 2 h before a rotating table (40 rpm) was switched on. Explants were allowed to incubate for an additional 24 h at 37 °C before transfer to 96-well plates containing 0.1 mL of culture medium with 0.4 mg/mL of luciferin. The plate was transferred to the modular incubator chamber rotating at 37 °C. A rapid gassing with 95% O_2/5% CO_2 was done in the first 5 min of a 15 min incubation period. Luciferase expression was quantified as bioluminescence photon counts using a cryogenically cooled Xenogen IVIS imaging system (Xenogen Corporation, Alameda, CA) coupled to a data acquisition computer at the Center of Molecular Imaging, University of Michigan. A digital gray scale image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photon counts emerging from active luciferase within the wells. Signal intensity was quantified as the sum of all detected photons within the region of interest during 1 or 3 min luminescent integration periods. Results were expressed as fold photon counts compared to untransfected explants present in the plate. For each experiment each virus was added to 2 explants.

Caco-2 Cell Culture. Caco-2 cells (ATCC HTB37) of passage 33−44 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated FBS, 0.1 mM nonessential amino acids (NEAA), 45 nM vitamin E, and 100 units/mL penicillin and streptomycin until confluent (typically 3 days). Medium was changed to DMEM with 5% FBS, 0.1 mM NEAA, 45 nM vitamin E, 0.1 μM selenium (Na_2SeO_3), 3 μM zinc ($ZnSO_4 \cdot 7H_2O$), and 100 units/mL penicillin and streptomycin, until the end of the experiment (typically 2 weeks). Cells were grown at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity.

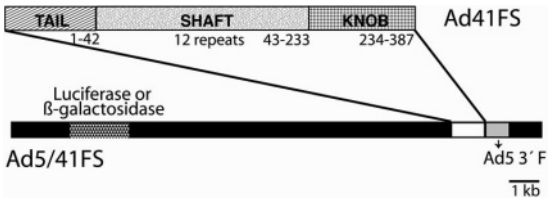

Figure 1. Genome organization of the chimeric Ad5/Ad41 (Ad5SHORT) vector. The genome of Ad41 short fiber is incorporated in place of the endogenous Ad5 fiber on the Ad5 backbone.

Adenoviral Transduction Studies in Caco-2 Cells. Caco-2 cells were seeded at a density of 4×10^5 cells/well on porous polycarbonate cell culture inserts with a pore size of 0.4 μm and a surface area of 0.9 cm^2 in 12-well plates or in tissue culture treated multiwell plates. Medium was changed every other day for 2 weeks in both apical and basolateral chambers. In certain cases, monolayers were mechanically wounded by cutting the monolayer with a cell scraper along one diameter of the insert. Cells were washed with DMEM to remove cell debris, and feeding medium was added to both apical and basolateral chambers. Cells were kept in culture for up to 48 h to allow for wound healing to occur. For adenoviral transduction studies, basolateral medium was renewed while apical medium was replaced with medium containing the desired adenoviral vector(s) at a MOI (multiplicity of infection; number of viral particles/cell) of 10, 100, and 1000. After 2 h incubation at 37 °C, medium was replaced with vector-free medium and cells were incubated for an additional 48 h at 37 °C. At this time, β-galactosidase expression was assayed with X-gal staining while luciferase expression was assayed by bioluminescence imaging following a protocol similar to the one used for the intestinal biopsies.

X-Gal Staining. β-Galactosidase expression was visualized with X-gal staining following established protocols. Cells were studied under an Olympus inverted light microscope (model CK).

Data Analysis. Statistical significance was determined using Student's *t* test. *P* values less than 0.05 were considered statistically significant. Data are shown as mean ± standard error of mean.

Results

Ad5SHORT Vector Preparation and Characterization. Chimeric Ad5/Ad41 vectors (Figure 1), which expressed the Ad41 short fiber, were constructed via homologous recombination in *E. coli* BJ5183 (*recBC sbcBC*). We were able to

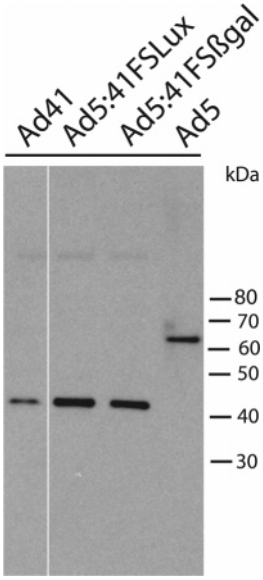

Figure 2. Western blot analysis of the Ad5/Ad41 chimeric vectors Ad5SHORT-luc and Ad5SHORT-CMV. Ad5 and A41 are included as reference. Western blotting was performed with the R72 polyclonal antibody that cross-reacts with the fiber of Ad5 and the short fiber of Ad41. Ad5 fiber size is 60.6 kDa, and Ad41 short fiber size is 41.4 kDa.

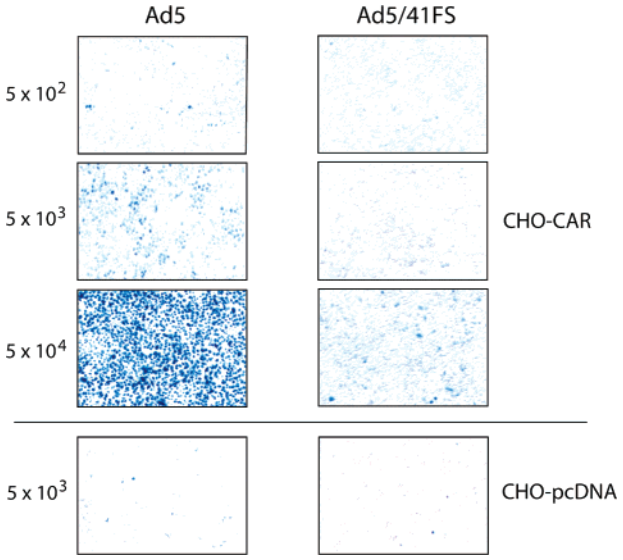

Figure 3. Transduction of CAR-positive (CHO-CAR) and CAR-negative (CHO-pcDNA) CHO cells by Ad5 and Ad5/Ad41FS (Ad5SHORT) β-galactosidase expressing vectors, visualized by X-gal staining. Ad5 transduction increases with vector concentration (left panel). Application of the chimeric Ad5SHORT vector results in significantly lower transduction levels (right panel). Both vectors fail to transduce CAR-negative CHO-pcDNA cells (bottom row).

isolate the chimeric Ad5/Ad41 vector after transfection into C7 cells expressing Ad5 polymerase and preterminal protein. The titers of Ad5SHORT-betagal and Ad5SHORT-luc were found to be 1.24×10^{11} PFU/mL and 1.32×10^{11} PFU/mL, respectively, compared to 1.62×10^{11} PFU/mL and 1.67×10^{11} PFU/mL for the parent Ad5CMV-betagal and Ad5CMV-luc, respectively. These corresponded to particle:PFU ratios of 32 and 30 for the Ad5SHORT-betagal and Ad5SHORT-luc vectors, respectively. These values are comparable to the particle:PFU ratio of 27 obtained for the parent Ad5 vectors, and are in the range reported by others.[23]

Purified particles were separated by SDS−PAGE and blotted on a polyvinylidene fluoride membrane, which was used for Western blot analysis. Western blotting was performed with the R72 polyclonal antibody which was isolated against Ad2 fiber but cross-reacts with Ad5 fiber and Ad41 short fiber. The results of the Western blot analysis for the parent vector Ad5 and the chimeric vectors Ad5SHORT-luc and Ad5SHORT-betagal are shown in Figure 2. Ad41 is also included as reference. No band corresponding to the Ad5 fiber (60.5 kDa) was observed after Western blot analysis of Ad5SHORT-luc and Ad5SHORT-betagal. A band corresponding to the Ad41 short fiber at 41.4 kDa was the only band observed for both chimeric vectors. This confirms the substitution of Ad5 fiber with the Ad41 short fiber on the chimeric vectors' capsids.

Ad5 and Ad5SHORT Transduction in CHO Cells. Ad5 and Ad5SHORT vectors were applied at increasing concentrations to transfected CHO expressing human CAR (CHO-CAR) to study interaction of the viruses with the CAR receptor. As seen in Figure 3, a dose-dependent increase in transduction was observed for the Ad5 vector with high transduction levels seen at 50 000 PFU. Application of the non-CAR binding Ad5SHORT-betagal vectors resulted at low transduction levels at all vector concentrations tested, confirming the absence of a CAR-binding fiber. Both vectors failed to transduce CAR-negative CHO-pcDNA cells.

Ad5 and Ad5SHORT Transduction Studies in Human Intestinal Explants. Dramatic differences were observed in transduction efficiencies of Ad5 and Ad5SHORT vector after application to intestinal explants of normal and ulcerative colitis patients (Figure 4). Quantified as photon counts (fold control) by biophotonic imaging, administration of Ad5-SHORT in normal explants resulted in 23-fold reduction in transduction efficiency compared to the parent Ad5 vector (6.17 ± 4.68 vs 141.83 ± 22.96, $n = 3$, $p < 0.05$). In explants from ulcerative colitis patients, Ad5SHORT transduction levels were at 11.29 ± 4.64 photon counts (fold control) compared to 186.5 ± 43.43 for the Ad5 vector ($n = 7$, $p < 0.01$).

Ad5 and Ad5SHORT Transduction Studies in Differentiated and Undifferentiated Caco-2 Cells. As previously reported,[34,35] transduction of Caco-2 cells by adenoviral vectors was low. Application of the Ad5SHORT vectors led to further significant decrease in transduction efficiency.

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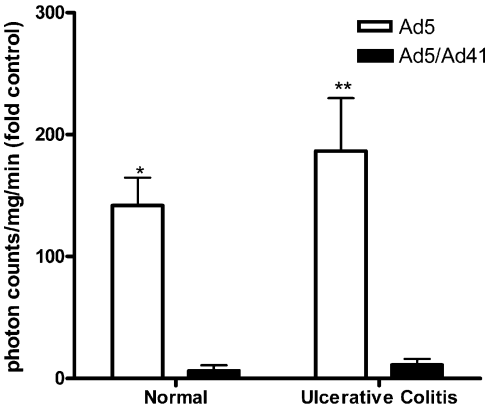

Figure 4. Transduction of human intestinal explants from healthy and ulcerative colitis patients by Ad5 and Ad5/Ad41 (Ad5SHORT) luciferase expressing vectors, quantified by bioluminescence imaging. Ad5 vector application resulted in significantly higher transduction levels in both healthy ($p <$ 0.05) and UC ($p < 0.01$) patients. Results are expressed as fold control differences in photon counts mg^{-1} min^{-1} compared to untransfected explants. $N = 3$ for normal and $N = 7$ for UC patients.

When viral vectors were applied on the apical side of Caco-2 cells, transfection with the Ad5SHORT vector was 3.9-fold (MOI 1000), 4.2-fold (MOI 100), and 2.5-fold (MOI 10)

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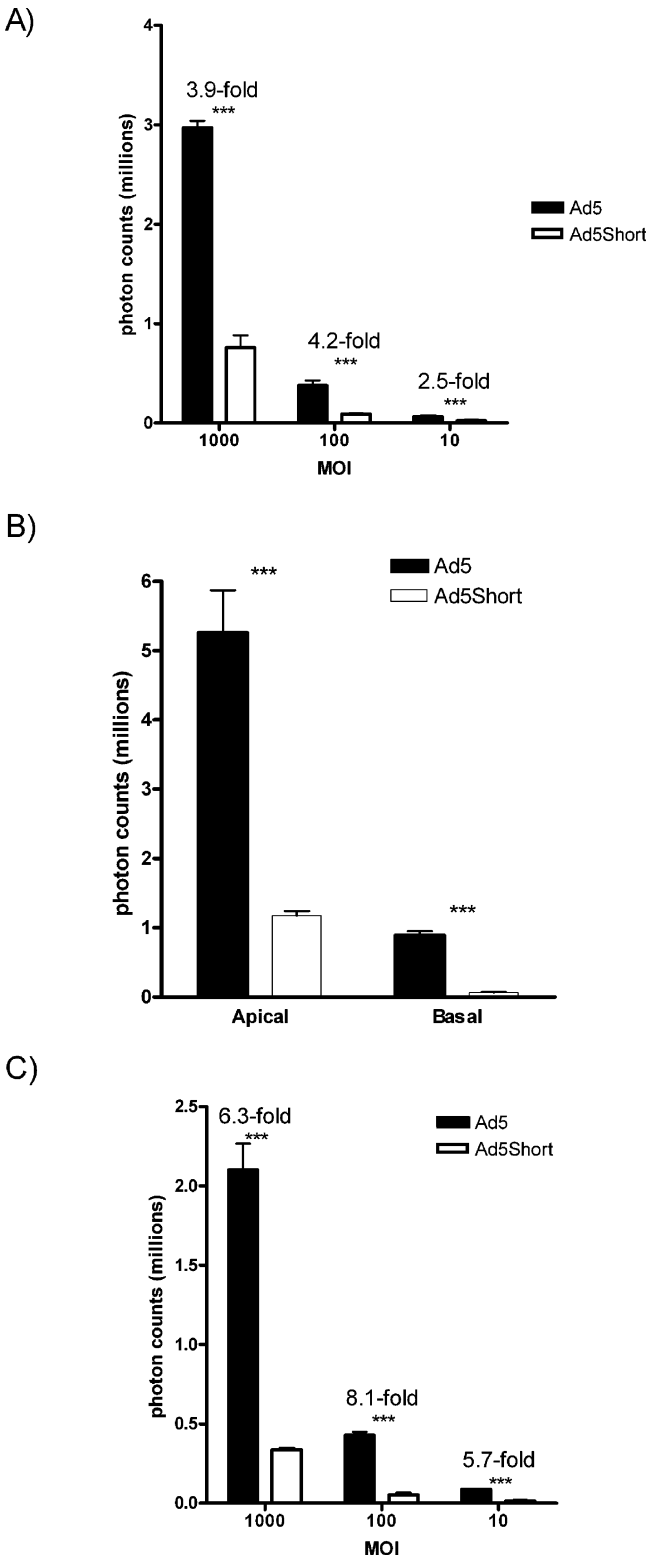

Figure 5. Differences in Ad5 and Ad5SHORT transduction of differentiated and undifferentiated Caco-2 monolayers: (A) differentiated Caco-2 monolayers grown on tissue culture plates (photon counts ± SD, $n = 3-4$); (B) apical and basolateral application on differentiated Caco-2 monolayers grown on transwell inserts (photon counts ± SD, $n = 3-4$); (C) undifferentiated Caco-2 cells on tissue culture plates (photon counts ± SD, $n = 3-4$). *** denotes $p < 0.001$.

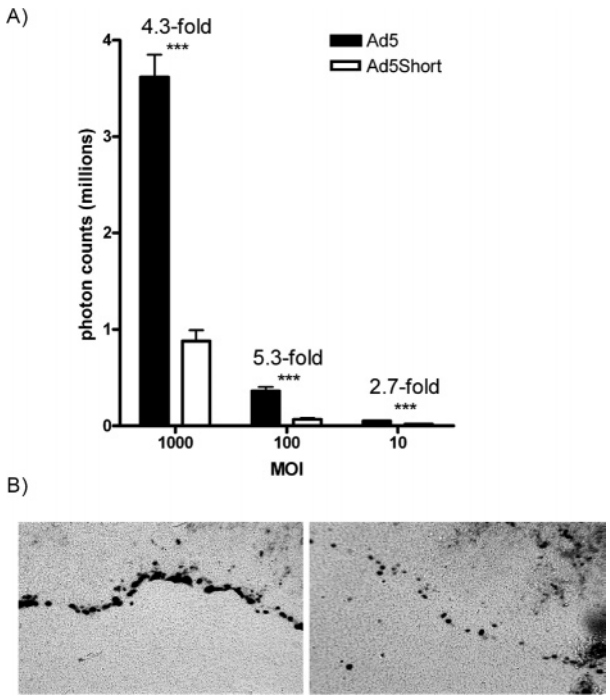

Figure 6. Differences in Ad5 and Ad5SHORT transduction of wounded Caco-2 monolayers. (A) Differences in bioluminescence activity 48 h after Ad5CMV-luc and Ad5SHORT-luc application. Results are expressed in photon counts ± SD ($n = 3-4$). *** denotes $p < 0.001$. (b) X-gal staining 48 h after application of Ad5CMV-betagal (left) or Ad5SHORT-betagal (right) on wounded Caco-2 monolayers.

lower than the one observed with the Ad5 vector (Figure 5A). Interestingly, this effect was enhanced when viruses were applied on the basolateral side of the cells, as a 13.8-fold reduction of transfection efficiency was observed (MOI 1000) (Figure 5B). In undifferentiated Caco-2 cells, a 6.3-fold (MOI 1000), 8.1-fold (MOI 100), and 5.7-fold (MOI 10) reduction in transduction efficiency was observed with the Ad5SHORT vector (Figure 5C).

Ad5 and Ad5SHORT Transduction Studies in Wounded/Migrating Systems. We compared differences in transfection by Ad5 and Ad5SHORT of repairing (wounded) Caco-2 monolayers. Ad5SHORT administration resulted in reduction of transduction levels by 4.3-, 5.3-, and 2.7-fold for the MOIs of 1000, 100, and 10 respectively (Figure 6A). X-gal staining of monolayers transfected with vectors carrying the LacZ reporter gene confirmed the above differences (Figure 6B). Transfection with the parent (Ad5) vector results in almost complete transduction of the cells on the migrating edge at the highest MOI of 1000. However, when the modified vector was used, a significantly lower number of cells were transfected. A similar pattern was observed with lower MOIs (data not shown).

Discussion

Genetically modifying widely used adenoviral 5 vectors, by substituting the fiber protein with that of other serotypes, is a common way to manipulate vector tropism.[15,24−33] The goal is typically to reduce the natural Ad5 hepatic tropism while, at the same time, increasing affinity of the vectors for other cell types of interest. In the former case, the engineered vectors can be further manipulated to achieve retargeting through a bispecific conjugate.[11] Pseudotyped vectors can also be useful tools in studying virus−cell interactions. We engineered a recombinant Ad5 vector to express the short fiber of Ad41 and were able to produce the vector at high titers on C7 cells. The chimeric, non-CAR binding vector exhibited dramatically reduced transduction efficiency of human intestinal epithelial cells both in vitro with Caco-2 cell cultures and ex vivo with intestinal biopsy cultures.

Serotype 41 adenoviruses are enterotropic adenoviruses (subgroup F) with some distinct characteristics compared to the most commonly used Ad5 vectors. The most notable is the presence of two distinct fibers on the viral capsid, a long one (60.5 kDa) that binds CAR and a shorter one (41.4 kDa) that lacks CAR binding abilities.[14] The function and receptor for the short fiber remain unknown. Due to the difficulties associated with growing subgroup F viruses, incorporating the Ad41 fibers on the capsid of Ad5 vectors provides an alternate way to produce gene delivery vectors with Ad41 tropism as well as a tool to study the functions of Ad41 fibers. Two recent studies have been published on incorporating the Ad41 fibers in the Ad5 backbone. Schoggins et al.[25] constructed chimeric Ad5 viruses containing either one or both of the Ad41 fibers. Although the Ad41 fiber was the dominant fiber on chimeric vectors carrying both Ad41 fibers, the authors were unable to isolate a vector expressing only the Ad41 short fiber. Ad5 vectors carrying only the Ad41 long fiber produced transduction levels similar to those produced by the parent Ad5. Ad5/Ad41 tandem vectors showed a slight reduction, compared to Ad5, in transduction efficiency of A549, Caco-2, and IM-9 cells as well as a more significant drop in liver transduction after retroorbital administration in mice. Furthermore, chimeric vectors expressing Ad41 short and Ad7 fibers showed a dramatic drop in transduction efficiency both in vivo and in vitro compared to Ad5. More recently, Nicol et al.[15] reported the construction of an Ad5 recombinant vector carrying the Ad41 short fiber using PCR gene overlap extension and propagation on 633 and subsequently PerC6 cells. The chimeric Ad5/Ad41 vector showed dramatically reduced transduction levels, less than 5% of that of the parent Ad5, in ARL-6 rat hepatocytes and RGE rat endothelial cells in vitro, as well as diminished liver sequestration after intravenous delivery to rats.

We were able to engineer a recombinant Ad5 vector expressing the short fiber of Ad41, providing an alternative production method to the one proposed by Nicol et al.[15] to produce this Ad5/Ad41 chimeric vector. We utilized homologous recombination in *E. coli* BJ5183 (*recBC sbcBC*) to produce the chimeric DNA backbone and C7 cells expressing Ad5 polymerase and preterminal protein to propagate the virus. This system allowed for the production of chimeric vectors at high titers ($1.24-1.32 \times 10^{11}$ PFU/mL). These titers are similar to the ones obtained for the parent Ad5 vector, suggesting that the construct production

in C7 cells may contribute to higher production levels.[21] When tested in HEK 293 cells, the chimeric vectors retained the infectivity of the parent Ad5 vector, evident by the comparable particle:PFU ratios between the two vectors. Western blot analysis (Figure 2) confirmed the absence of the Ad5 fiber on the chimeric vectors (lack of 60.5 kDa band) and its substitution with the Ad41 short fiber (band at 41.4 kDa). Unlike Ad5 fiber, the short fiber of Ad41 does not bind to CAR.[14] Lack of CAR binding ability of the chimeric vectors was demonstrated in CHO cells expressing CAR (CHO-CAR, Figure 3). The parent Ad5 vectors successfully transduced CHO-CAR cells in a dose-dependent manner as has been documented in the literature.[4] However, significantly reduced transduction levels were observed for the recombinant Ad5/Ad41 vector (Figure 3, left panel). The significant decrease in transduction cannot be attributed to reduced vector viability as comparable infectivity for the two vectors was obtained in the HEK 293 cells. Both parent and recombinant vectors resulted in low transduction levels in CAR-negative control CHO-pcDNA cells although CHO K1 cells express both α_v integrins and heparin sulfate glucosaminoglycans.[7] The transduction levels of the chimeric vectors in CHO-pcDNA cells were similar to the ones obtained with CHO-CAR cells, further supporting lack of CAR binding for this vector. The data from the CHO-pcDNA cells suggest that receptors other than CAR play a minor role in transduction in vitro. Although it has been suggested that the Ad41 short fiber might not possess HSG binding properties either,[15] the data from the CHO and CHO-CAR cells suggest that ablation of CAR binding is the reason for the low transduction efficiency of the chimeric vectors. A less dramatic drop in transduction efficiency would be expected if other receptors, such as HSG, contributed significantly to viral attachment and entry.

Due to the enterotropic nature of Ad41, we studied transduction efficiency of the chimeric vector in ex vivo intestinal biopsy cultures and in in vitro cell cultures of Caco-2 cells. The human intestinal explant culture offers the opportunity to study interactions of adenoviral vectors and intestinal epithelial cells in the context of normal tissue components and organization and avoids the issue of species differences associated with testing agents in animal models. Caco-2 cells offer a "clean" in vitro screening system for the study of interactions between viral vectors and enterocytes. We included in our studies biopsies from patients with ulcerative colitis and in vitro models of mucosal wound healing. The latter two were selected since inflammatory bowel disease tissue represents a potential target for gastrointestinal gene delivery.[17,18] Furthermore, since the function and receptor of the short fiber of Ad41 remain unknown, we were interested in the effect its presence, as the sole fiber, would have on transduction efficiency. Ad41 has been reported to exhibit higher affinity for enterocytes than Ad5.[34]

In all intestinal epithelial models tested, we noticed significant reduction in transduction levels with the recombinant vector compared to the parent Ad5. In cultures of intestinal biopsies the differences were 23-fold for healthy patients and 16.5-fold for ulcerative colitis patients (Figure 4). It is worth mentioning that, in the human intestinal explant studies, transduction mainly occurs in a subset of enterocytes with a flattened dedifferentiated phenotype, present at the edge of the biopsy.[35] Similar differences were observed in the Caco-2 system with the recombinant vector producing approximately 4-fold lower transduction levels compared to Ad5 (Figure 5A) at all MOIs tested. Schoggins et al.[25] reported a similar reduction in transduction levels comparing their tandem Ad5/Ad41 vectors to Ad5. When the vectors were applied on the basolateral cell surface (Figure 5B), undifferentiated cells (Figure 5C), or a model of mucosal wound healing (Figure 6), this difference was even more pronounced. Interestingly, both vectors exhibited higher apical than basolateral transduction. Higher apical transduction has been previously reported for Caco-2 cells with the parent Ad5 vector.[36]

Our data show that replacement of Ad5 fiber with Ad41 short fiber significantly reduces transduction efficiency of primary intestinal epithelial cells and of fully differentiated Caco-2 cells. Schoggins et al.[25] reported similar transduction levels in differentiated Caco-2 cells comparing Ad5 and an Ad5/Ad41 recombinant vector carrying the Ad41 long fiber and a small reduction with their Ad5/Ad41 tandem vector (short fiber in excess). On the basis of the results from both studies it appears that the higher affinity for enterocytes associated with Ad41[34] cannot be attributed to the function of one of the fiber proteins and may be associated with other unique capsid elements. However, one cannot exclude the possibility that the ratio of short to long fiber significantly affects binding properties. In Ad41 viruses this ratio has been reported at 1:1.[13] With the currently available data, it is unclear whether Ad41 short fiber plays any role in transduction. It has been hypothesized that it might represent a natural, if redundant, mutant.[14]

Our data show a significant reduction in transduction by the Ad5SHORT vector in the intestine, the target tissue of Ad41 virus. Previous reports on modified Ad5 vectors carrying the Ad41 short fiber by Nicol et al.[15] and Schoggins et al.[25] also showed dramatic reduction in transduction of the chimeric vector in several different cell lines (rat hepatocytes,[15] rat endothelial cells,[15] A549 cells,[25] and IM-9 cells[25]) as well as a significant drop in liver transduction.[15,25] While this drop in transduction is desirable in the design of retargeted vectors that could be further manipulated for targeting via bispecific antibodies, future work should focus in demonstrating that these vectors can regain infectivity when targeted at specific tissues and cells. Plaque forming assays with HEK 293 cells performed with Ad5SHORT suggest that the vector retains the infectivity of the parent Ad5.

In conclusion, we were able to engineer, in high titers similar to the parent vectors, an Ad5 recombinant vector expressing the short fiber of Ad41 as the sole fiber on the

(36) Walter, E.; et al. Adenovirus mediated gene transfer to intestinal epithelial cells as a potential approach for oral delivery of peptides and proteins. *J. Controlled Release* **1997**, *46*, 75−87.

capsid. We report, for the first time, transduction results of a chimeric Ad5/Ad41 vector in human intestinal tissue, the natural target tissue of the fiber-"donor" virus, Ad41. CAR-binding ablation resulted in significant reduction of transduction levels of intestinal epithelial cells both in the intestinal biopsy culture system and in vitro utilizing Caco-2 cell cultures. The same results were obtained in inflammatory bowel disease tissue, a potential target for gastrointestinal gene therapy. These studies highlight the potential for using this chimeric Ad5/Ad41 vector as a scaffold for the development of retargeted adenoviral vectors. Finally, our results suggest that the short fiber does not appear to be mediating, at least by itself, the increased enterocyte affinity of Ad41.

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