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Adenoviral targeting using genetically incorporated camelid single variable domains

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Abstract

The unique ability of human adenovirus serotype 5 (Ad5) to accomplish efficient transduction has allowed the use of Ad5-based vectors for a range of gene therapy applications. Several strategies have been developed to alter tropism of Ad vectors to achieve a cell-specific gene delivery by employing fiber modifications via genetic incorporation of targeting motifs. In this study we have explored the utility of novel anti-human carcinoembryonic antigen (hCEA) single variable domains derived from heavy chain (VHH) camelid family of antibodies to achieve targeted gene transfer. To obtain anti-CEA VHHs we produced a VHH-display library from peripheral blood lymphocytes RNA of alpacas at the peak of immune response to the hCEA antigen. We genetically incorporated an anti-hCEA VHH into a de-knobbed Ad5 fiber-fibritin chimera and demonstrated selective targeting to the cognate epitope expressed on the membrane surface of target cells. We report that the anti-hCEA VHH employed in this study retains antigen recognition functionality and provides specificity for gene transfer of capsid-modified Ad5 vectors. These studies clearly demonstrated the feasibility of retargeting of Ad5-based gene transfer using VHHs.

Keywords

Adenovirus; Targeted gene transfer; Single variable domain; Carcinoembryonic antigen; single domain antibody

Adenoviral vectors embody a number of unique attributes which has allowed their use for a wide range of gene transfer applications (1, 2). Despite this utility, the parental tropism of the Ad5 widely employed for vector applications embodies some critical limits. In this regard, a relative paucity of the primary adenovirus (Ad) receptor coxsackie-and-adenovirus receptor (CAR) renders some cells/tissues resistant to infection via Ad5-based vectors.

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Alternatively, the native distribution of CAR has mitigated against the pharmacologic goal of cell-specific gene transfer with use of Ad in *in vivo* delivery contexts.

On the basis of these considerations, strategies have been developed to alter Ad tropism to make feasible cell specific targeting using both molecular adapter proteins and genetic capsid modifications (3). In the first instance, the method of Ad5 targeting based on bi-specific adapters has allowed specific delivery using a range of relevant cellular markers. Molecular adapters have consisted of chemically coupled antibody (Ab)-ligand fusions, diabodies, as well as genetic fusions between ligand or single-chain variable fragments (scFvs) and the ectodomain of the CAR. To this end, bispecific molecular adapters have allowed modification of Ad tropism and key proof-of-principle demonstrations of targeted gene transfer in both *in vitro* and *in vivo* delivery contexts (4-7).

A number of considerations, however, have recommended that such strategies to modify Ad tropism be accomplished in the context of “single unit” configurations, an approach at odds with the two component Ad vector-plus-adapter method. On this basis, methods to alter Ad tropism have capitalized on the knowledge that select viral capsid proteins including hexon, pIX and fiber are the key determinants of vector tropism. Whereas a wide range of targeting moieties have been employed for recombinant Ad vectors (reviewed in (8)), the restricted repertoire of available targeting peptides functionally compatible with fiber insertion have led to the consideration of antibody (Ab) species for Ad retargeting purposes. Such an approach could logically exploit the large repertoire of available Ab targeting reagents and the facile methods to generate new specificities using biopanning methodologies. Furthermore, antibody-based retargeting offers the potential of targeted delivery for cell infection specificity rather than the less precise tropism expansion embodied in the peptide ligand methods. Importantly, the ability to genetically engineer Abs allows additional flexibility in their utility for Ad retargeting for an ultimate human application.

Modification of Ad tropism using genetic incorporation of Ab ligands requires the capacity to re-engineer the fiber protein to incorporate large/complex Ab species. Furthermore, the biosynthesis of candidate Ab species designed for Ad incorporation must be compatible with Ad capsid protein synthesis and assembly. Unfortunately, to this point, available Ab species have not proved to be biologically compatible with cytosolic Ad capsid synthesis and assembly resulting in loss of binding affinities. This loss of binding specificity, in the instance of incorporated scFv, is likely due to the fact that Ad capsid proteins are normally synthesized in the cytosol with assembly in the nucleus, while scFv molecules are typically routed through the rough endoplasmic reticulum. In this context, the redox state of the cytosol likely results in improper scFv folding which perturbs the structural configuration required for Ag recognition, leading to our observations of loss of binding specificity. Despite the demonstrated utility of “stabilized” scFv with molecular scaffold motifs designed to resist the deleterious effect of the cytosol redox state for Ad retargeting (9, 10), the limited available repertoire of target specificities of this class of scFv practically restricts this approach.

On the basis of these deliberations we have considered the utility of alternate Ab species which might embody a stability profile compatible with the cytosolic biosynthesis of Ad

capsid proteins. The discovery of unconventional immunoglobulins (Ig) derived from the serum of animals in the camelid family (camels and alpacas) that consist of only the two heavy-chains (no light-chains) as the basis of antigen (Ag) recognition and binding has made possible their use for Ad-mediated gene therapy (11). Camelid heavy-chain-only Abs (hcAbs) possess characteristics ideal for an Ad retargeting strategy: (1) cytosolic stability allowing functional incorporation into the Ad capsid and (2) compatibility with phage biopanning selection to allow target cell specificity (8). Unlike conventional Ig, hcAbs contain a single variable domain (VHH) linked to two constant domains (C_H2 and C_H3) (12). Cloned and isolated single domain antibodies (sdAbs) have shown a remarkable stability profile compared to conventional lgs and scFvs. In addition, engineered sdAb fusion proteins have demonstrated effective targeting in model systems (13, 14). Based on these useful attributes, a number of groups have developed non-immune sdAb libraries which have been employed for successful biopanning in order to define useful targeting specificities (15-19). Recently, modifications of Ad5 capsid proteins were evaluated for utility in Ad retargeting by using pIX protein as a platform for the presentation of scFv or sdAb molecules. However, due to the nature of the Ad capsid proteins synthesis and virion assembly, even the ER-targeted pIX-scFv proteins were incorporated into the Ad capsid at a very low level which was not sufficient to retarget virus infection. In contrast, it was shown that expression of anti-EGFRvIII sdAb on the Ad capsid through fusion to pIX can be used to redirect Ad infection (20).

We have thus here endeavored a proof-of-principle study to evaluate the utility of sdAb as a candidate Ab for Ad retargeting. In this study we have explored the utility of novel VHHs derived using a VHH-display library from peripheral blood lymphocytes RNA of alpacas at the peak of their immune response to the human carcinoembryonic antigen (hCEA) to achieve targeted Ad-mediated gene transfer. Our results validate targeted gene delivery using Ad vector with capsid incorporated VHH. This finding provides an important technical approach allowing practical linkage of capsid modification of Ad5 vector and ligand-based strategies for targeting gene delivery.

MATERIALS AND METHODS

Immunization of alpacas with CEA protein

Purified human carcinoembryonic antigen (hCEA) protein (ProNique Scientific, Castle Rock, CO) was used to immunize alpacas (*Vicugna pacos*) in alum/CpG adjuvant as previously described (21). Briefly, two adult male alpacas were given six immunizations at three-week intervals, each including multi-site subcutaneous injections containing a total of 100 μg of hCEA in the pre-scapular region. Serum at the completion of the immunization process contained Ab titers for hCEA exceeding $1{:}1 \times 10^4$ in both alpacas.

Identification of anti-CEA VHHs

The VHH-display library was prepared from B cells obtained from the alpacas four days following the final boost with hCEA. A single VHH-display phage library was prepared using RNA from both alpacas. Library construction, panning, phage recovery and clone fingerprinting were performed as previously described (22, 23), including recent

improvements (24). Approximately 6×10^6 independent clones were obtained and pooled to yield the VHH-display phage library. The hCEA protein was coated onto Nunc Immunotubes (Nunc, Rochester, NY) for panning. Following two panning cycles, >80% of the selected clones recognized hCEA on enzyme-linked immunosorbent assay (ELISA) (signals >4-fold over background). The 38 clones producing the strongest signals were characterized by DNA fingerprinting (24) and identified nine unique VHHs. DNA sequencing of these clones identified four hCEA-binding VHH families that appeared completely unrelated. The best VHH representatives of the four families (JJB-A3, JJB-B2, JJB-B5 and JJB-D1) were expressed as thioredoxin fusion proteins (25), purified and further characterized. Dilution ELISAs were performed to assess the apparent affinity (EC_{50}) of each purified VHH as previously described (23). Nunc Maxisorb plates (Nunc) were coated overnight at 4°C with 1 μg per ml human CEA protein (Abcam, Cambridge, MA). Subsequently, plates were blocked in binding buffer containing 5% w/v non-fat milk (LabScientific, Livingston, New Jersey) in Tris-buffered saline (TBS). The blocking buffer was then replaced with a dilution series of JJB-A3, JJB-B2, JJB-B5 or JJB-D1 in binding buffer with 0.05% Tween 20. Plates were incubated at 25°C for one hour and then washed three times with TBS. Bound VHHs were detected with HRP/anti-E-tag mAb (Bethyl Laboratories, Montgomery, TX).

Cells and reagents

The murine colon adenocarcinoma MC38 cells has been described previously (26). MC38CEA cells expressing hCEA were generated by retroviral transduction with hCEA cDNA. MC38 and MC38CEA cells were provided by Dr. H.R. Herschman (University of California, Los Angeles, CA). 293F28 cells expressing wild-type Ad5 fiber protein have been described previously (27). The human embryonic kidney HEK293 cells were purchased from Microbix Biosystems (Ontario, Canada); human colorectal adenocarcinoma LS174T cells, prostate adenocarcinoma PC-3 cells and lung cancer A549 cells were originally obtained from ATCC (Manassas, VA). The Chinese hamster ovary CHO cells and CHOCAR cells expressing hCAR as well as 293F28 cells were provided by Dr. H. Ugai (Washington University in St. Louis, St. Louis, MO) and mouse Lewis lung carcinoma cells were provided by Dr. H. Yan (Washington University in St. Louis, St. Louis, MO). All cells were cultured in DMEM/F12 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO) and cultured at 37°C in a humidified atmosphere with 5% CO_2.

Anti-hCEA VHH clone C17 was obtained from a semi-synthetic llama (*Lama glama)* sdAb library as described previously (28) and provided by A. Hayhurst (Texas Biomedical Research Institute, San Antonio, TX). Anti-epidermal growth factor receptor (EGFR) VHH clone VHH122 has been described previously (29) and provided by E.F. Patz Jr. (Duke University Medical Center, Durham, NC).

Flow cytometry

To determine the levels of hCAR and hCEA expression cells were seeded at 5×10^5 cells per well in 6-well tissue culture plates and allowed to grow overnight. The next day, the wells were washed with PBS, cells were collected, and subjected to fluorescence-activated

cell sorter (FACS) analysis. Chinese hamster ovary CHO and CHO-CAR cells were evaluated for hCAR expression using anti-CAR mAb (RmcB, 100 ng per ml, Millipore, Billerica, MA) and an anti-mouse AlexaFluor 488-labeled goat IgG at 100 ng per ml (Molecular Probes, Eugene, OR). For evaluation of hCEA expression, cells were stained with anti-human CEA rabbit IgG (Millipore) and anti-rabbit fluorescein isothiocyanate (FITC)-labeled goat IgG (Millipore). Mouse IgG1 negative control (Millipore) and rabbit IgG isotype control (Thermo Scientific, Rockford, IL) were used as isotype controls. Cells were incubated with Abs for one hour at 4°C. Following incubation with secondary Abs, the cells were collected, washed three times in FACS buffer and approximately 1×10^4 cells were illuminated at 488 nm and fluorescence was detected in the FITC (525/20 nm) channel. Data were analysed using FlowJo software (Tree Star, Ashland, OR).

To determine the efficacy and specificity of the hCEA-targeted VHHs binding to hCEA expressed on the cell surface, MC38 and MC38CEA cells were incubated with 100 ng per ml of JJB-A3, JJB-B2, JJB-B5 or JJB-D1 VHHs for 30 min at 25°C. Cells then were washed one time with PBS, incubated with anti-E-tag FITC-conjugated goat Ab for one hour at 4°C, and subjected to FACS analysis as described above.

Adenoviral vectors

Replication incompetent *E1*-deleted Ad5 vectors were created using a two-plasmid rescue method. The chimeric fiber-fibritin protein contained the N-terminal Ad5 fiber tail region fused to the entire fibritin protein with the trimerizing foldon domain of bacteriophage T4 following by Gly-Gly-Gly-Gly-Ser peptide linker connected to the VHH open reading frame (ORF) as described previously (30). To generate a PCR product encoding a fragment of the VHH ORF clone B2: BamH1-B2 (TTA GGA TCC CAG GTG CAG CTC GTG) and B2-Swa1 (GGG ATT TAA ATA ATT GTG GTT TTG GTG); clone C17: BamH1-C17 (AAA GGA TCC GAA GTC CAA CTG GTT G) and C17-Swa1 (TTT ATT TAA ATC AGG CCG CCG ACG A); clone VHH122: BamH1-VHH122 (AGA GGA TCC GAG GTG CAA CTG C) and VHH122-Swa1 (CCC ATT TAA ATC ATG AGG AGA CGG TG) primers were used. The PCR product was cloned into a plasmid pKan556FF using *BamH* I and *Swa* I sites to generate the pKan566FF-B2, pKan566FF-C17 and pKan566FF-VHH122, respectively. Insertion sequences then were confirmed by using restriction enzyme mapping and partial sequence analysis. Results of multiple amino acid sequence alignment of VHH domain of a camelid heavy-chain-only Ab are presented in Figure 1. The shuttle plasmids were digested using *EcoR* I and *Sal* I enzymes and integrated into the Ad5 genome by homologous recombination in the *Escherichia coli* strain BJ5183 with pVK700 plasmid comprised of the human cytomegalovirus (CMV) major immediate-early enhancer/promoter element coupled to the firefly luciferase (Luc) gene. The recombinant viral genomes with VHH-fiber-fibritin fusions were linearized with *Pac* I and then transfected into 293F28 cells stably expressing the native Ad5 fiber cells (27) using SuperFect Transfection Reagent (Qiagen, Chatsworth, CA). After additional round of amplification on 293F28 cells, the viruses were amplified in HEK293 cells and purified twice by CsCl gradient centrifugation and dialyzed against 10 mM HEPES, 1 mM $MgCl_2$, pH 7.8 with 10% glycerol as previously described (31). To verify inserted modifications of the fiber gene all viral genomes were subjected to partial sequencing analysis. The concentration of physical viral particles (vp) was determined by

measuring absorbance of the dissociated virus at A_{260} nm using a conversion factor of 1.1×10^{12} vp per absorbance unit (32).

Enzyme-linked immunosorbent assay (ELISA)

Nunc Maxisorb plates (Nunc) were coated overnight at 4°C with hCEA protein (Abcam) diluted at a concentration of 1 μg per ml in 50 mM carbonate buffer (pH 8.6). The unsaturated surface of the wells was then blocked for one hour at 25°C by the addition of 200 μl of blocking buffer including TBS with 5% w/v non-fat milk (LabScientific). The blocking buffer was replaced with 100 μl of Ad diluted in binding buffer (TBS with 0.05% Tween 20 and 5% w/v non-fat milk). Plates were incubated at 25°C for one hour and then washed three times with washing buffer (TBS with 0.05% Tween 20). Bound viral particles were detected by incubation for one hour at 25°C with 100 ng per ml of polyclonal anti-adenovirus goat Ab, (ViroStat, Portland, ME). The wells were washed three times with washing buffer and then anti-goat rabbit IgG conjugated with horseradish peroxidase (HRP) (Dako Corporation, Glostrup, Denmark) at 250 ng per ml were added and incubation was continued for one hour. The color was developed with Sigma FAST *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, Saint Louis, MO) as recommended by the manufacturer.

Western blotting

Samples were preincubated in Laemmli sample buffer at 99°C for 10 min and separated using a 4-20% gradient polyacrylamide Precise Protein gel (Thermo Scientific). For electrophoresis under seminative condition, samples were not boiled. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and the blots were developed with SIGMA FAST 3,3′-diaminobenzidine system (Sigma) according to the manufacturer's protocol using anti-Ad5 fiber tail mAb (4D2, 200 ng per ml, NeoMarkers, Fremont, CA) and goat anti-mouse Ig-HRP at 500 ng per ml, (DakoCytomation Denmark A/S, Glostrup, Denmark) for Ad fiber protein detection.

Thermostability *in vitro*—LS174T cells were plated in 24-well tissue culture plates in triplicate at a density of 5×10^4 cells per well, allowed to adhere and grow overnight. The next day, cells were infected with Ad vectors at 5×10^3 vp per cell in DMEM/F12 with 2% FBS for one hour, then culture medium was removed and fresh media containing 10% FBS was added to each well. Forty-eight hours after infection cells were lysed and Luc activity was analyzed as described below. The relative infectivity was obtained by changing the relative light units (RLU) readings of the heat-treated viruses to the percentage of the readings of untreated viruses.

RNA preparation and RT-PCR assay

The levels of hCEA mRNA expression in cells were determined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from 1×10^7 cells using RNeasy Mini Kit (Qiagen), following standard protocol, and quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The first-strand cDNA was synthesized using random hexamer primers with an Omniscript RT kit (Qiagen) and used as the template for PCR. The following primers were used: hCEAf206: 5′-CCA CCA CTG CCA AGC TCA CTA-3′; hCEAr388: 5′-CTG GGG TAG CTT GTT GAG TTC CTA-3′ (amplicon 183 bp).

After the initial denaturation (5 min at 95°C), amplification was performed with 30 cycles of 30 sec at 95°C, 20 sec at 62°C and 35 sec 72°C. The glyceraldehyde 3-phosphate dehydrogenese (GAPDH) cDNA was used as an internal standard for template loading of PCR by using the following primers: for human GAPDH: forward: 5′-TCC CAT CAC CAT CTT CCA-3′; reverse: 5′-CAT CAC GCC ACA GTT TCC-3′ (amplicon 388 bp) and for murine GAPDH: forward: 5′-GGA GTC CAC TGG CGT CTT CAC-3′; reverse: 5′-GAG GCA TTG CTG ATG ATC TTG AGG-3′ (amplicon 165 bp). PCR was performed under the following conditions: after the initial denaturation (5 min at 95°C) 30 cycles (95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min). The hCEA gene specific qPCR template standard (OriGene Technologies, Rockville, MD) was used as an internal control for PCR primers. PCR products were analyzed by 1% agarose electrophoresis with ethidium bromide staining.

Gene transfer assay

Cells were seeded at 5×10^4 cells per well in 24-well tissue culture plates and allowed to grow overnight. The next day, cells were washed one time with PBS, and then infected with 5×10^3 vp per cell of Ad vectors in triplicate. After one hour, cell culture media was removed, the wells were washed with PBS and fresh media was added. After 48 hours, cell culture media was removed, and cells were washed one time with PBS, and lysed. Luciferase activity was analyzed as described below.

Expression of recombinant Ad5 knob

The knob domain of Ad5 fiber protein was expressed in *Escherichia coli* as described previously (33). Soluble His-tagged Ad5 knob was purified by gravity-flow affinity chromatography using a Ni-NTA resin (Qiagen). The concentration of the purified protein was determined using DC Protein Assay (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Purified recombinant protein was evaluated by Western blot using anti-His mAb (HIS-1, 1:2000, Sigma) and and goat anti-mouse Ig-HRP at 500 ng per ml (DakoCytomation).

Competitive inhibition of gene transfer

CHO and CHO-CAR cells were seeded at 1×10^5 cells per well in 24-well tissue culture plates and allowed to grow overnight. The next day, cells were washed one time with PBS, and incubated for one hour at 4°C with serial dilutions of Ad5 fiber knob protein or bovine serum albumin (BSA). After incubation, cells were washed with PBS, and infected with 5×10^3 vp per cell of Ads for one hour at 37°C. Subsequently, cell culture media was removed, cells were washed with PBS, and fresh media was added.

MC38, MC38CEA and LS174T cells were seeded at 1×10^5 cells per well in 24-well tissue culture plates and allowed to grow overnight. For hCEA mediated inhibition of gene transfer Ads were preincubated with hCEA or BSA at different concentration at 25°C for 30 min. Cells then were washed one time with PBS, and infected with Ads at 5×10^3 vp per cell. After incubation for one hour at 37°C cell culture media was removed, cells were washed with PBS and fresh media was added. After 48 hours, cell culture media was removed, cells were washed one time with PBS, and lysed. Luciferase activity was analyzed as described below.

Luciferase assay

The Luciferase Assay System (Promega, Madison, WI) and ORION microplate luminometer (Berthold Detection Systems, Oak Ridge, TN) were used for the evaluation of Luc activity of infected cells. Luciferase activity was normalized by the protein concentration of the cell lysate using DC Protein Assay (Bio-Rad), according to the manufacturer's instructions. Data are expressed as relative light units (RLU) per mg of total protein.

Statistical analysis

All error terms are expressed as the standard deviation of the mean. Significance levels for comparison of differences between groups in the experiments were analyzed by Student's *t* test. All reported *p*-values are two-sided. The differences were considered significant when *p*-value was < 0.05.

RESULTS

Isolation the anti-hCEA VHH

A VHH phage display library was prepared representing the VHH repertoire from two alpacas immunized with purified hCEA protein and screened to identify VHHs that bind to hCEA. Four VHHs (JJB-A3, JJB-B2, JJB-B5, JJB-D1) representing apparently unrelated hCEA-binding VHH groups were selected and characterized for hCEA affinity by dilution ELISA (Figure 2A) and the half maximal effective concentrations (EC_{50}) values representing the VHH concentration that produced 50% maximum signal on the ELISAs were calculated. These results indicated that JJB-A3, JJB-B2 and JJB-D1 bound hCEA with the EC_{50} of approximately 0.15, 0.2 and 1 nM, respectively, while JJB-B5 had much lower affinity for hCEA ($EC_{50} \sim 50$ nM).

To characterize hCEA expression profile in a panel of cancer cells levels of hCEA mRNA expression was evaluated using RT-PCR. For this study we determined endogenous expression of hCEA mRNA in human and mouse cancer cells using RT-PCR. As shown in Figure 2B, PC-3 and MC38CEA cells demonstrated high levels of hCEA mRNA expression in comparison with other tested cells, whereas murine MC38 and Lewis lung carcinoma cells which were used as negative control showed the lowest levels of hCEA mRNA expression. Also, hCEA protein expression on the cell surface of MC38 (hCEA-) and MC38CEA (hCEA+) murine colon cancer cells and human prostate adenocarcinoma PC-3 cells, colorectal adenocarcinoma LS174T cells and lung cancer A549 cells were evaluated by FACS analysis (Table 1). The relative levels of hCEA expression varied in different cell lines and correlated well with their levels of hCEA mRNA expression (Figure 2B). Human cancer cells demonstrated high (PC-3), mediate (LS174T) and low (A549) basal levels of hCEA expression (Table 1). MC38CEA cells demonstrated higher number of hCEA expressing cells and the mean value of fluorescence intensity in comparison with MC38 cells.

The four VHHs were also characterized by FACS for their ability to recognize hCEA expressed on the surface of mammalian cells. For this study MC38CEA and MC38 cells were selected based on CEA expression and results of studies demonstrated the utility of

MC38CEA cells for targeted Ad-mediated gene delivery using anti-CEA scFv (6, 34). As shown in Figure 2C, JJB-A3 and JJB-B2 both clearly recognized cells expressing hCEA. There was an increased number of hCEA+ cells which bound JJB-A3 and JJB-B2 VHHs (78 and 80%, respectively), and JJB-B2 was selected for further studies.

Recombinant Ad vectors

For this study we developed a panel of recombinant Ad5-based vectors expressing the firefly luciferase (Luc) gene under transcriptional control of the human cytomegalovirus (CMV) major immediate-early enhancer/promoter element (Figure 3A). For AdB2Luc, the chimeric fiber-fibritin protein coding DNA containing the N-terminal Ad5 fiber tail region was fused to DNA encoding the entire fibritin protein with the trimerizing foldon domain of bacteriophage T4 following by a Gly-Gly-Gly-Gly-Ser peptide linker connected to VHH clone JJB-B2 (30). Llama VHH clone C17 directed against nonconventional epitopes of tumor-associated CEA was obtained from a semi-synthetic VHH library, using RNA from peripheral blood lymphocytes of llama immunized with recombinant purified soluble CEA (28). Vectors expressing anti-EGFR VHH122 (29) fused to fiber-fibritin (AdVHH122Luc) and wild-type Ad5 fiber (Ad5Luc) were used as isogenic control and wild-type control Ad vector, respectively.

To demonstrate the incorporation of the targeting VHH-fiber-fibritin fusion proteins into the virus, 5×10^9 vp of boiled and unboiled purified Ads were loaded in each lane and subjected to SDS-PAGE followed by Western blot analysis using anti-fiber mAb. Predicted molecular weight (MW) of fiber monomers are 61.6 kDa for Ad5Luc; MW 68.4 kDa for AdC17Luc; MW 67.7 kDa for AdB2Luc and MW 67.8 kDa for AdVHH122Luc. As shown in Figure 3B, genetic incorporation of VHHs produced stable fusion with fiber-fibritin molecules that maintained trimerization potential of VHH-fiber-fibritin chimeric proteins under native conditions.

Next, we tested how modifications in the capsid proteins affect the structural integrity of the Ad virion by comparing the thermostability of fiber-modified Ad vectors to wild-type Ad5Luc. Based on results of preliminary study that demonstrated temperature-dependent decreasing of Ad-mediated gene transfer (35), Ad5Luc, AdB2Luc, AdC17Luc and AdVHH122Luc viruses were preincubated at 42°C for different times before the infection of LS174T cells, and relative gene transfer efficiency was obtained by comparing with the gene transfer of unheated Ads. As shown in Figure 3C, there was a time-depended decreasing of gene transfer efficiency of all heat-treated Ad vectors, and about 40% of Luc expression was retained even after a 60-min incubation at 42°C.

Binding properties of the Ad vectors to the hCEA

To evaluate specificity of binding recombinant Ad vectors to hCEA, purified AdB2Luc and AdC17Luc vectors displaying an anti-hCEA VHH-fiber-fibritin chimera, AdVHH122Luc expressing anti-EGFR VHH-fiber-fibritin or Ad5Luc with wild-type fiber protein were incubated with the hCEA protein adsorbed onto a surface 96-well ELISA plate (Figure 4A). Results of ELISA using anti-fiber mAb revealed a significant degree of binding of AdB2Luc

and AdC17Luc expressing anti-hCEA VHH-fiber-fibritin to the hCEA in contrast to AdVHH122Luc and Ad5Luc which demonstrated no binding to the hCEA.

Based on results of initial screening using ELISA the AdB2Luc was selected for further evaluation transduction efficiency and target specificity. Cancer cells demonstrated varied basal levels of hCEA expression were infected with Ad vectors displaying the different VHH-fiber-fibritin chimeras or Ad5Luc with wild-type fiber. Since all tested Ad vectors comprise identical CMV promoter Luc gene cassettes, Ad transduction was compared by evaluation of Luc expression in the infected cells. Forty-eight hours after infection, cells were harvested and Luc expression was analyzed. Levels of Luc expression varied in the different cell lines in proportion to viral doses of infection (results not shown). As illustrated in Figure 4B, infection with AdB2Luc yielded lower Luc expression compared to Ad5Luc, and the relative levels of Luc expression in cancer cells correlated well with their levels of hCEA mRNA expression (Figure 2B) and with hCEA protein expression on the cell surface of cancer cells (Table 1). Additionally, to quantify effects of Ad fiber modifications on the relative levels of Luc expression we used the AdB2Luc-to-Ad5Luc reporter gene expression ratio. AdB2Luc-to-Ad5Luc ratios of Luc expression were calculated for each cell line with the RLU normalized by the total protein concentration of the cell lysate. The AdB2Luc-to-Ad5Luc ratio was 2.5×10^{-2} for PC-3 cells, 1.4×10^{-2} for LS174T cells, 1.1×10^{-3} for A549 cells, and 6.0×10^{-4} for Lewis lung carcinoma cells.

Specificity of the Ad-mediated gene transfer

Next we investigated whether AdB2Luc and AdC17Luc vectors displaying the two different anti-hCEA VHHs on fiber-fibritin retain specificity for the appropriate CEA expressing (CEA+) cells. Based on results of initial screening (Table 1) MC38 (hCEA-) and MC38CEA (hCEA+) murine colon cancer cells were selected for further evaluation transduction efficiency and target specificity. To evaluate the specificity of Ad mediated gene transfer, MC38 (Figure 5A) and MC38CEA (Figure 5B) cells were infected with AdB2Luc, AdC17Luc, AdVHH122Luc and Ad5Luc; and the level of Luc reporter gene expression was assessed 48 hours after infection. As shown in Figure 5C, infection with AdB2Luc produced about a ~56-fold increase ($P<0.01$) in reporter gene expression in hCEA-positive MC38CEA cells in comparison with MC38 cells. In contrast, Luc expression was only slightly (~5-fold; $P<0.05$) increased in hCEA+ cells following AdC17Luc infection. There were no significant differences across Luc expression in tested cells infected with AdVHH122Luc and Ad5Luc, isogenic and wild-type control Ads, respectively. We used the AdB2Luc-to-Ad5Luc and AdC17Luc-to-Ad5Luc ratios to quantify the relative levels of Luc expression in hCEA− MC38 and hCEA+ MC38CEA cells. The AdB2Luc-to-Ad5Luc ratio was 4.3×10^{-3} for MC38 cells, 3.7×10^{-1} for MC38CEA cells, and the AdC17Luc-to-Ad5Luc ratio of Luc expression was 2.8×10^{-3} for MC38 cells, 8.6×10^{-3} for MC38CEA cells.

CAR-independent AdB2Luc infection

Next we evaluated whether modification in the Ad5 fiber resulted in the ability of AdB2Luc to achieve CAR-independent binding and infection *in vitro*. Since previous studies demonstrated the ability of recombinant Ad5 knob to block binding of the corresponding Ad5 vectors or recombinant fiber protein to its receptor (33), we expressed recombinant Ad5

knob and evaluated the purified proteins by Western blotting with anti-His mAb (data not shown). For this analysis, we evaluate the hCAR expression in hCAR-expressing CHO-CAR (36) and CHO Chinese hamster ovary cells using FACS. As shown in Table 1, CHO-CAR cells demonstrate high level of hCAR expression in comparison with CHO cells. To investigate whether an AdB2Luc vector displaying an anti-hCEA VHH-fiber-fibritin produces CAR-independent infection, hCAR-expressing CHO-CAR and CHO cells (lacking hCAR) were pretreated with different concentrations of recombinant Ad5 knob or BSA at one hour prior to infection with AdB2Luc or Ad5Luc. As shown in Figure 6A, preincubation with of Ad5 knob did not inhibit AdB2Luc-mediated Luc gene expression in CHO-CAR cells. In contrast, Ad5Luc infection was efficiently inhibited by recombinant Ad5 knob protein in a dose-dependent manner (Figure 6B). Incubation hCAR expressing cells with 10, 50 and 200 μg per ml of Ad5 knob resulted in 50, 75 and 85% decreased Luc expression following infection with Ad5Luc, respectively. As expected, there was no blocking effect of incubation of wild-type hCAR negative CHO cells with recombinant Ad5 knob for both Ad5Luc- and AdB2Luc-mediated gene transfer in the same experiment (Figure 6A and Figure 6B).

Dose-depended inhibition of AdB2Luc gene transfer by hCEA

Additionally, to confirm a specificity of AdB2Luc infection we evaluated hCEA-mediated inhibition of Luc gene transfer. For this study human colon cancer LS174T cells were used as a positive control for hCEA expression (37). AdB2Luc was preincubated with different concentration of hCEA or BSA for 1 hour before infection of MC38 and MC38CEA mouse colon cancer cells and LS174T cells. Forty-eight hours after infection, cancer cells were lysed and Luc activity was measured (Figure 6C). Results of a gene transfer blocking assay demonstrated a dose-depended inhibition of Luc gene transfer in both CEA+ cell lines following pretreatment of AdB2Luc with hCEA. Gene transfer efficiency of AdB2Luc was significantly reduced after incubation with blocking protein, and only 24% and 30% of Luc expression was retained following infection of MC38CEA and LS174T cells, respectively, after incubation with 1.5 μg per ml of hCEA. In contrast, preincubation of AdB2Luc with hCEA protein at the highest concentration did not affect in Ad-mediated gene transfer in the hCEA negative MC38 cells.

Taken together, our data demonstrate that an AdB2Luc vector with a genetically incorporated anti-hCEA VHH in a de-knobbed Ad5 fiber-fibritin chimera retains hCEA recognition functionality and provides specificity of gene transfer of capsid-modified Ad.

DISCUSSION

In this study, we evaluated the utility of employing a VHH recognizing hCEA for targeting of Ad-mediated gene delivery. To develop CEA-targeted recombinant Ad5-based vectors, we genetically incorporated an anti-hCEA VHH into a de-knobbed Ad5 fiber-fibritin protein. We found that anti-hCEA VHHs employed in this study did not disrupt the trimerization capability of the Ad fiber and the antigen (Ag) recognition of the anti-hCEA VHHs were retained. In addition, we demonstrated the ability of an anti-CEA VHH fused to

fiber-fibritin chimera to provide specific and efficient targeting of CEA-expressing cancer cells for Ad-mediated gene transfer.

The CEA, an oncofetal glycoprotein, is normally expressed at low levels in gastrointestinal epithelium and overexpressed in a wide range of human carcinomas, including colon, rectum, breast, lung, and pancreas (38-41). Importantly, CEA overexpression is often associated with development of metastatic diseases (42-44). As a membrane tumor-specific Ag, CEA can be used for imaging or therapeutic approaches. Preclinical studies have shown the up-regulation of CEA mRNA and protein expression in clinical tumor samples as well as human cancer cell lines following radiation treatment (45-47). The expression profile of CEA makes this protein an attractive tumor-cell specific target for Ad-mediated gene therapy. To this end, several studies have utilized the molecular adaptor retargeting approach in conjunction with anti-CEA scFv to demonstrate the feasibility of targeted Ad-mediated gene delivery and the utility of Ab-based targeting moieties to achieve selectivity of gene transfer using tropism-modified Ad (34). However, for clinical applications, a genetic modification of the Ad capsid is the preferred configuration for a targeted Ad vectors.

Recent studies validated the utility of fiber-based targeting moieties using synthetically constructed monobodies representing single domain antibody mimics based on the tenth human fibronectin type III domain (10Fn3) scaffold to achieve selectivity of gene transfer using tropism-modified Ad (48). In contrast to these synthetically constructed monobodies, we explored the utility of novel VHHs against hCEA derived from the Camelid family. These molecules possess unique structural features (11, 12, 14) potentially commensurate with Ad5 biosynthesis, assembly and targeting. Due to their single domain nature, engineered VHH fusion proteins are expected to be more resistant to aggregation and degradation than conventional scFvs (49) and have affinities and specificities comparable to scFvs, thus resulting in effective tumor targeting (13, 14). Regardless of the fact that camelid VHH molecules include putative disulfide bond sequences, VHHs are known to be resistant to cytosol-induced alterations due to stabilized folding properties (49). Recently, it was shown that expression of pIX-sdAb fusion protein on the Ad capsid resulted in retargeting of Ad infection in comparison with the ER-targeted scFv which was incorporated into the virion at a very low level, probably, due to cytosolic Ad capsid synthesis and assembly (20).

In this study we tested Ad fiber as the site for presentation of VHH-based targeting motifs on the surface of the Ad capsid and as an alternative approach of Ad retargeting by using pIX protein for the sdAb expression. This is based on the recognition that targeting moieties incorporated at alternate Ad capsid sites, including pIX, hexon or penton proteins have not provided the effective Ad retargeting achieved using fiber localization of target-specific proteins (20, 50, 51). For this study we produced a VHH-display library from peripheral blood lymphocytes RNA of alpacas at the peak of their immune response to the hCEA Ag. After several rounds of panning enrichment, initial screening and DNA fingerprinting, we obtained four unique VHHs positive for hCEA binding and with EC_{50}s in the range 50 nM to 150 pM. Due to the relatively larger size of VHH compared to phage defined peptides, we were required to radically re-engineer the fiber capsid protein using a “fiber replacement” approach that make feasible capsid incorporation of antibody-size molecules (30).

We genetically incorporated the VHH ORF into the C-terminus of de-knobbed fiber-fibritin chimera protein comprising the entire Ad5 fiber shaft fused to the 12th coiled-coil fragment of fibritin protein of bacteriophage T4. Initial evaluation of recombinant Ad vectors demonstrated that genetic incorporation of VHH-fiber-fibritin resulted in expression of trimeric fusion protein molecules on the surface of Ad particles. There was a chance that binding specificity of some of the VHHs might be altered due to the relatively large size of the chimeric VHH-fiber-fibritin protein. However, our results demonstrated selective binding of Ad vectors displaying the VHH clone B2 to the cognate epitope expressed on the surface ELISA plate or specific targeting of cancer cells with surface CEA.

In this study a panel human cancer cells was used for initial determination of transduction efficiency of human Ad serotype 5-based recombinant vectors including LS174T cells previously characterized for CEA expression (37) and murine Lewis lung carcinoma cells were used as negative control for expression of human CEA. Next, for evaluation of specificity of the Ad-mediated gene transfer and for dose-depended inhibition of AdB2Luc gene transfer by hCEA murine colon adenocarcinoma MC38 (CEA-) and MC38CEA (CEA +) cells were used based on results of studies demonstrated the feasibility of this cell model for evaluation of targeted Ad-mediated gene delivery utilized the molecular adaptor retargeting approach using anti-CEA scFv (6, 34).

In addition, the results of competitive inhibition studies confirmed CEA-dependent and CAR-independent AdB2Luc-mediated gene transduction. A panel of VHH-fiber-fibritin expressing Ad vectors tested in this study also included AdC17Luc as hCEA-targeted Ad control vector as well as AdVHH122Luc which was used as isogenic control vector. To develop AdC17Luc we used anti-hCEA VHH clone C17 obtained from a semi-synthetic llama sdAb library (28) and belongs like a VHH B2 to the VHH2 subfamily (52). In the initial study, sdAb C17, harboring a 6-His tag at the C-terminus, was purified by immobilized ion metal-affinity chromatography and demonstrated moderate affinity for soluble CEA with K_D ~8.3 nM using surface plasmon resonance, and hCEA specificity using flow cytometry of MC38CEA and LS174T cells (28). However, this sdAb demonstrated poor binding to the hCEA+ cells when expressed as a VHH-fiber-fibritin protein, possibly due to steric inhibition by the fiber-fibritin fusion partner. The VHH122 was produced using a llama-VHH domain library from peripheral blood lymphocytes RNA of llama immunized with a mixture of EGFR and EGFRvIII recombinant proteins and U87MG and ADLC-5M2 cell lysates. It was shown that VHH122 bound to both EGFR and EGFRvIII with roughly equal affinity, approximately 40 nM using surface plasmon resonance (29). In this study AdVHH122Luc expressing anti-EGFR VHH-fiber-fibritin was used as isogenic control vector, however, additional experiments, that are beyond the scope of this study, are required to investigate molecular mechanisms of AdVHH122 mediated gene transfer.

Our experiments sought to test the efficacy and specificity of Ad vectors displaying anti-CEA VHHs in the fiber protein for selective transgene expression. We developed a panel of recombinant Ad5-based vectors expressing a VHH-fiber-fibritin fusion protein. We showed that anti-hCEA VHHs employed in this study retain Ag recognition functionality and substantially modifies the cell-type specificity of gene transfer achieved using the capsid-

modified Ad5 vector. Thus, these proof-of-principal studies demonstrated feasibility of fiber modification using VHH to facilitate Ad retargeting.

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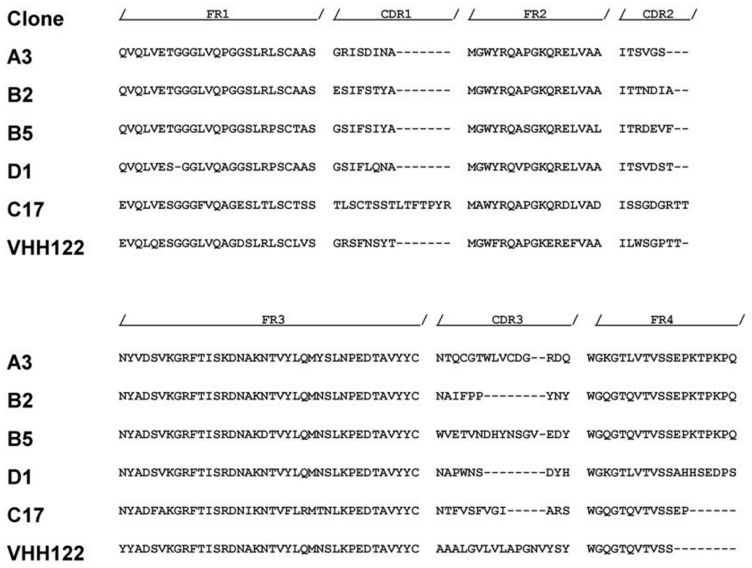
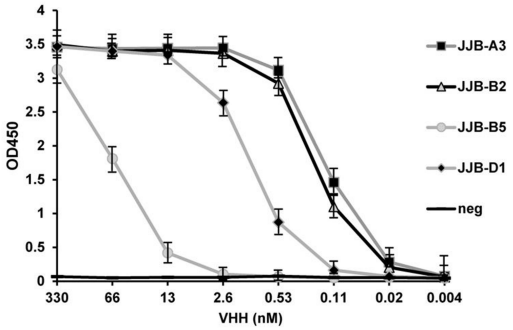


Figure 1.

Multiple amino acid sequence alignment of camelid VHH clones. Dashes indicate gaps introduced in order to optimize sequence alignment. VHH domain of a camelid heavy chain Abs clones B2, C17 and VHH122 were used for genetic incorporation into the chimeric VHH-fiber-fibritin. Predicted molecular weight (MW) of VHHs: A3: MW 13.8 kDa; B2: MW 13.4 kDa; B5: MW 14.4 kDa; D1: MW 13.2 kDa; C17: MW 14.1 kDa; VHH122: MW 13.5 kDa. FR1-4, framework regions; CDR1-3, complementarity determining regions.

A



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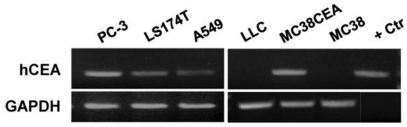

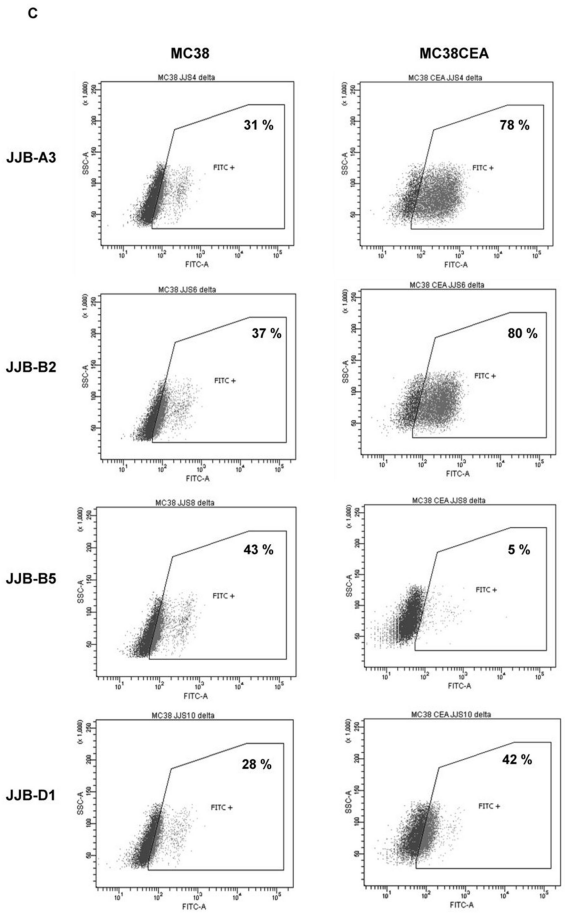

Figure 2.

Evaluation of anti-CEA VHH binding to hCEA protein. Each VHH was produced as soluble thioredoxin fusion protein in *Escherichia coli* cytosol. (A) Evaluation of VVH binding to hCEA protein by using dilution enzyme-linked immunosorbent assay (ELISA). The plates for ELISA were coated with purified hCEA protein and then purified VHH were added in wells at various concentrations. Bound VHHs were detected with HRP/anti-E-tag mAb. Each point represents a mean of six readings obtained in two separate experiments with the error bars showing standard deviations (s.d.). (B) The level of hCEA mRNA expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from human and mouse cancer cells, the first-strand cDNA was synthesized using random hexamer primers and used as the template for PCR. Products of PCR were analyzed by 1% agarose electrophoresis with ethidium bromide staining. One representative of three different experiments is shown. LLC, murine Lewis lung carcinoma cells; Ctr+, the hCEA gene specific qPCR template standard (OriGene Technologies). (C) Evaluation of efficacy and specificity of the CEA-targeted VHHs binding to hCEA expressed on the cell surface. MC38CEA and MC38 cells were incubated with 100 ng per ml of JJB-A3, JJB-B2, JJB-B5 and JJB-D1 VHHs. Bound anti-CEA VHHs were detected by using anti-E-tag FITC-conjugated goat Ab using FASC analysis. One representative of two different experiments is shown.

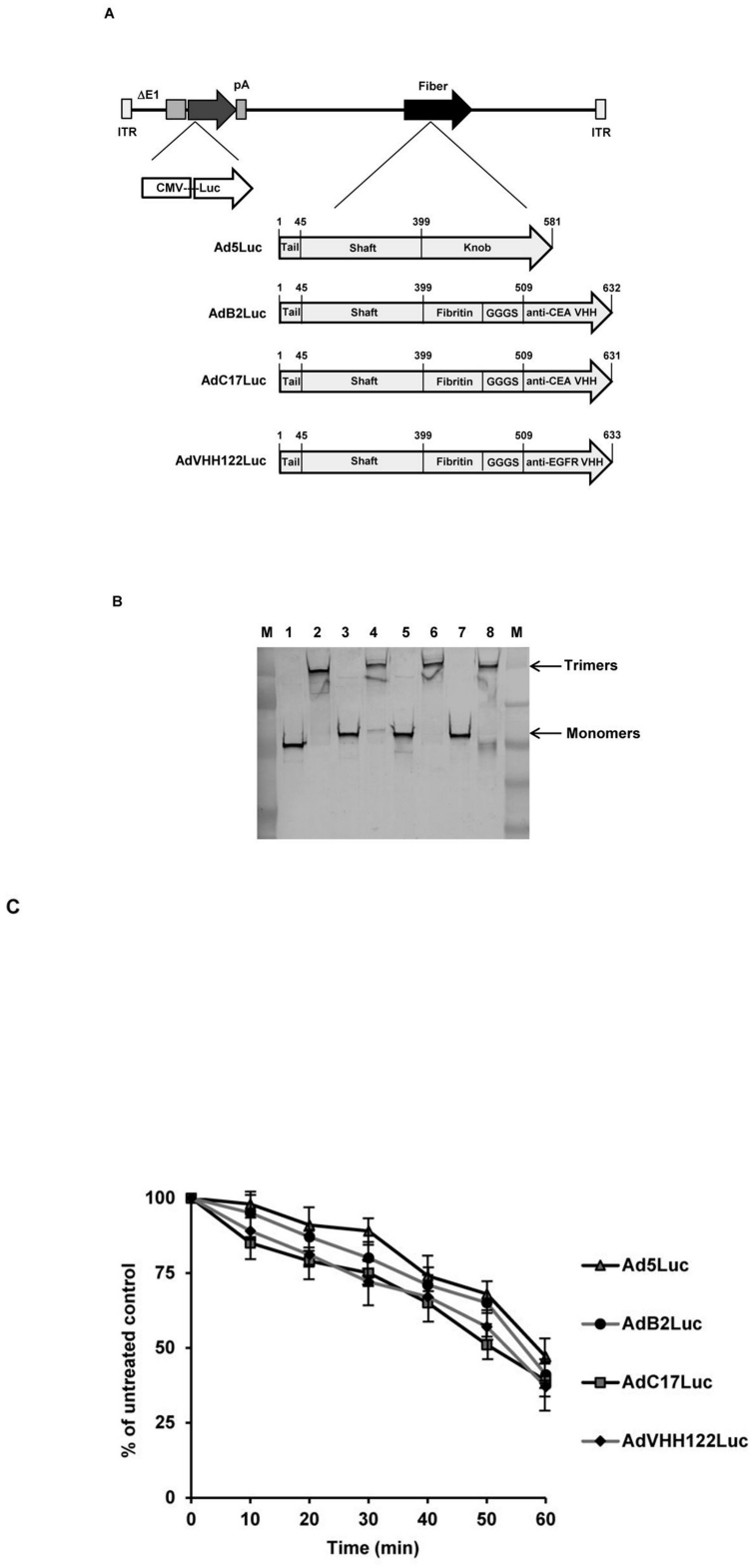

Figure 3.

Initial evaluation of Ad vectors expressing VHH-fiber-fibritin. (A). Simplified schematic of recombinant Ad vector genomes. Only genomic regions relevant to presented studies are highlighted. The knob domain of Ad5 fiber was removed and replaced with the trimerization domain of the T4 phage fibritin protein fused to the VHH using a flexible Gly-Gly-Gly-Ser linker. (B) Assessment of incorporation of VHH-fiber-fibritin proteins into Ad particles using Western blotting analysis. Equal amounts (5×10^9 vp) of purified the VHH-fiber-fibritin modified Ad vectors including AdC17Luc (lanes 3 and 4), AdB2Luc (lanes 5 and 6), and AdVHH122Luc (lanes 7 and 8) or the wild-type fiber Ad5Luc vector (lanes 1 and 2) were loaded in each lane with boiling in a sample buffer (lanes 1, 3, 5 and 7) or without boiling (lanes 2, 4, 6 and 8) and separated on SDS-PAGE followed by transfer to a PVDF membrane. Fiber protein expression was detected using anti-fiber mAb. (C) Thermostability of recombinant Ad vectors. The viruses were incubated at 42°C for different time intervals before the infection of LS174T cells. Forty-eight h after infection cells were subjected to Luc assay.

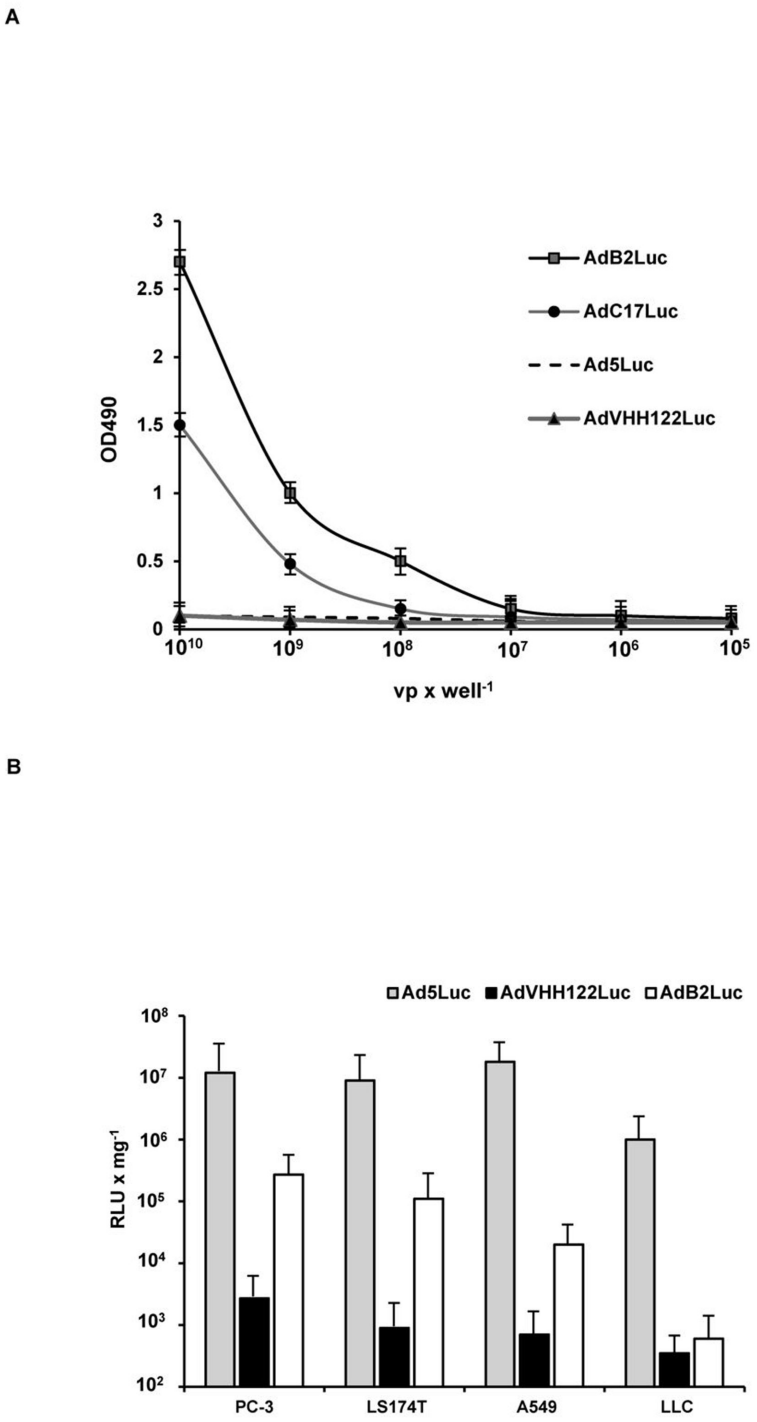

Figure 4.

Screening of binding properties of the recombinant Ad vectors. (A) Evaluation of Ad vectors binding to hCEA protein by using ELISA. The plates for ELISA were coated with purified hCEA protein and then purified Ad virions were added in wells at various concentrations. Bound viral particles were detected by using polyclonal anti-adenovirus goat Ab. Each point represents a mean ± s.d. of six readings obtained in two separate

experiments. (B) Evaluation of the specificity of AdB2Luc-mediated gene transfer. Human and mouse cancer cells were infected with 5×10^3 vp per cell of Ads. Luciferase activity was detected in the lysates of infected cells at 48 hours after infection. LLC, murine Lewis lung carcinoma cells. Data are presented as relative light units (RLU) per mg of total protein and bars represent the mean values ± s.d. of three independent experiments, each performed in six replicates.

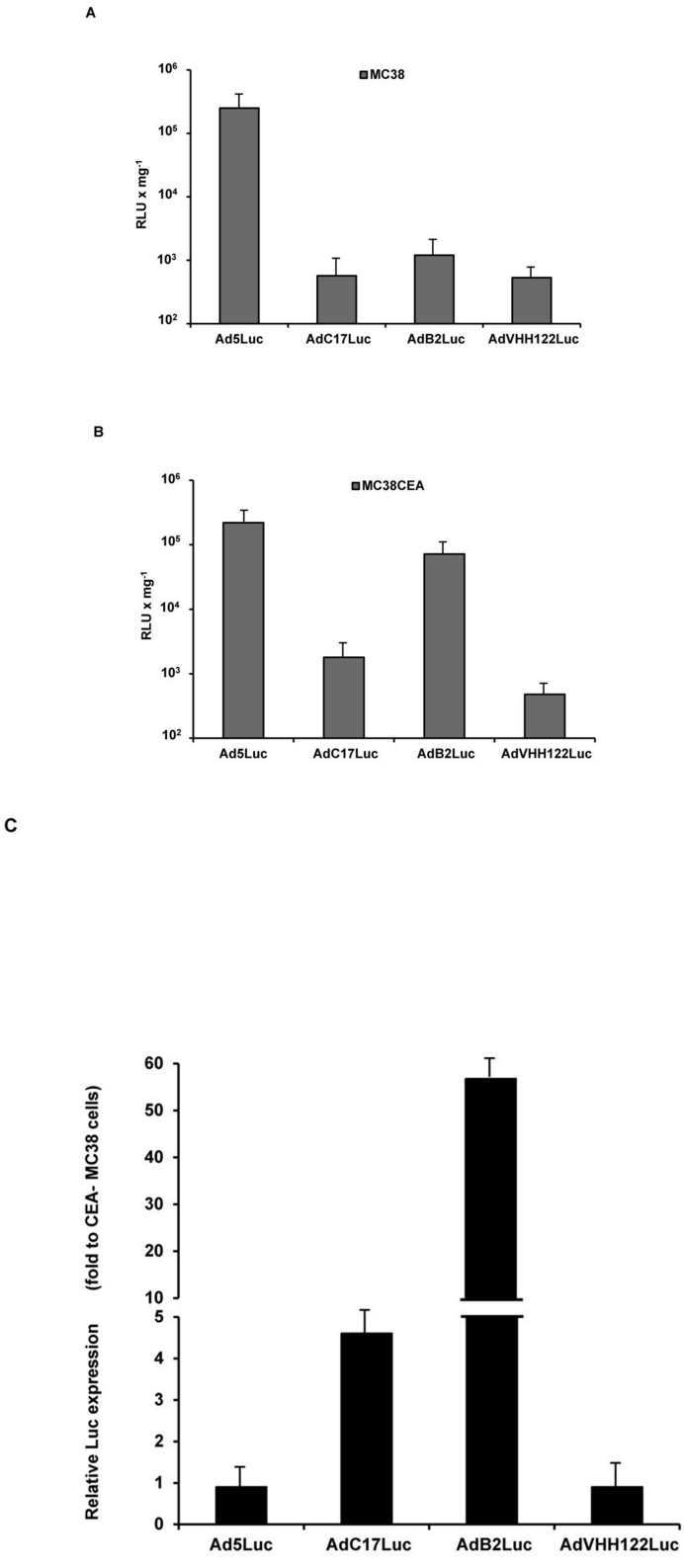

Figure 5.

Evaluation of efficacy and specificity of the CEA-targeted gene transfer. For evaluation of the efficacy of Ad-mediated reporter gene transfer, MC38 (A) and MC38CEA (B) cells were infected with 5×10^3 vp per cell of Ads. Luciferase activity was detected in the lysates of infected cells at 48 hours after infection. Data are presented as RLU per mg of total protein and bars represent the mean ± s.d. of four independent experiments, each performed in six replicates. (C) Ad targeting efficiency. The relative Luc expression was significantly increased in AdB2Luc infected MC38CEA cells in comparison with MC38 cells.

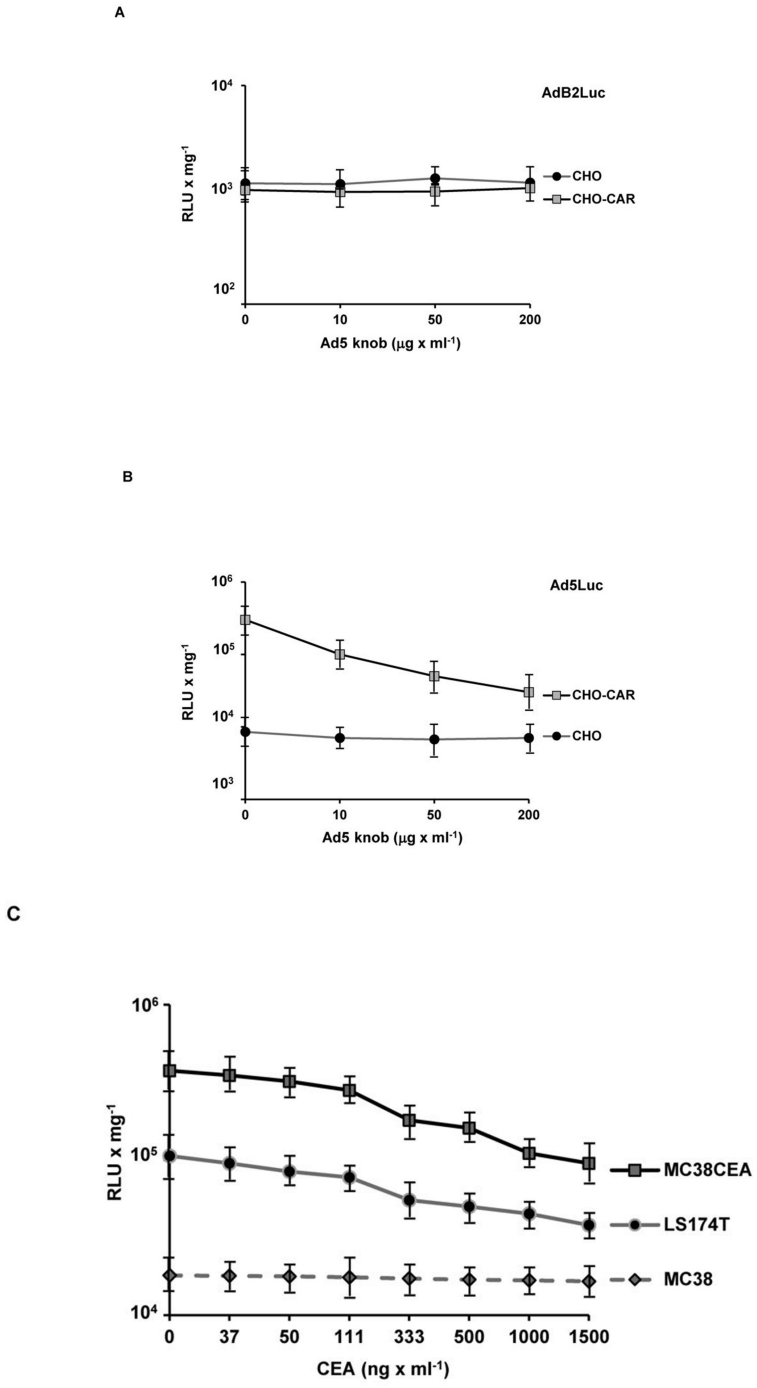

Figure 6.

AdB2Luc displaying an anti-hCEA VHH produces CAR-independent and CEA-dependent gene transfer. (A) CHO (hCAR-) and CHO-CAR (hCAR+) cells were preincubated with soluble Ad5 knob protein at different concentration or BSA and infected with 5×10^3 vp per cell of AdB2Luc. Luciferase activity was detected in the lysates of infected cells at 48 hours postinfection. Data are presented as RLU per mg of total protein and bars represent the mean ± s.d. of three independent experiments, each performed in six replicates. (B) Inhibition of

Ad5Luc-mediated gene transfer. CHO (hCAR-) and CHO-CAR (hCAR+) cells were preincubated with soluble Ad5 knob protein at different concentration or mock-treated and infected with 5×10^3 vp per cell of Ad5Luc. Luciferase activity was detected in the lysates of infected cells at 48 hours postinfection. Data are presented as RLU per mg of total protein and bars represent the mean ± s.d. of three independent experiments, each performed in four replicates. (C) Inhibition of AdB2Luc-mediated gene transfer. AdB2Luc was preincubated with hCEA or BSA at different concentration. MC38 and MC38CEA mouse colon cancer cells and LS174T human colon cancer cells were infected with AdB2Luc at 5×10^3 vp per cell. Luciferase activity was detected in the lysates of infected cells at 48 hours postinfection. Data points represent the mean RLU per mg of total protein ± s.d. of three independent experiments, each performed in six replicates.

Table 1

Flow cytometry analysis of hCEA and hCAR surface expression.

Cell line	% of positive cells (mean of fluorescence intensity)*	
	hCEA	hCAR
PC-3	53±9 (209±59)	93±8 (395±101)
LS174T	33±7 (224±34)	91±7 (683±126)
A549	6±4 (146±38)	98±8 (523±115)
MC38	2±3 (71±18)	N/A
MC38CEA	45±7 (178±31)	N/A
CHO	N/A	11±6 (121±29)
CHO-CAR	N/A	99±5 (2781±164)

* Human and mouse cancer cells, Chinese hamster ovary cells were stained with saturating amounts of Abs recognizing hCEA or hCAR and expression was evaluated by FACS analysis. A negative immunoglobulin G isotype primary Abs were used as a control. Data are the means ± s.d. of two or four independent experiments.