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Targeted Adenovirus Gene Transfer to Endothelial and Smooth Muscle Cells by Using Bispecific Antibodies

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A major hurdle to adenovirus (Ad)-mediated gene transfer is that the target tissue lacks sufficient levels of receptors to mediate vector attachment via its fiber coat protein. Endothelial and smooth muscle cells are primary targets in gene therapy approaches to prevent restenosis following angioplasty or to promote or inhibit angiogenesis. However, Ad poorly binds and transduces these cells because of their low or undetectable levels of functional Ad fiber receptor. The Ad-binding deficiency of these cells was overcome by targeting Ad binding to α_v integrin receptors that are sufficiently expressed by these cells. In order to target α_v integrins, a bispecific antibody (bsAb) that comprised a monoclonal Ab to the FLAG peptide epitope, DYKDDDDK, and a monoclonal Ab to α_v integrins was constructed. In conjunction with the bsAb, a new vector, AdFLAG, which incorporated the FLAG peptide epitope into its penton base protein was constructed. Complexing AdFLAG with the bsAb increased the β-glucuronidase transduction of human venule endothelial cells and human intestinal smooth muscle cells by seven- to ninefold compared with transduction by AdFLAG alone. The increased transduction efficiency was shown to occur through the specific interaction of the complex with α_v integrins. These results demonstrate that bsAbs can be successfully used to target Ad to a specific cellular receptor and thereby increase the efficiency of gene transfer.

Adenovirus has been widely used as a vector to deliver genes to a number of tissues in vivo, including lung, vascular, neuronal, and muscle tissue (7, 11, 16, 24). Attributes of the adenovirus system include its ability to deliver and express genes in nonproliferating cells, a well-characterized genome, and its ability to be grown to high titers. One potential improvement to this system would be to target the vector to receptors on specific cell types. This improvement would increase the efficiency of gene delivery to the target tissue and reduce nonspecific transduction of untargeted tissue, thus allowing lower vector doses to be administered with fewer unwanted side effects (7, 20). A further benefit of targeting adenovirus would be the increased transduction efficiency of tissues poorly transduced because of their low expression of adenovirus receptors (8).

In order to understand how to target adenovirus to new receptors and tissues, it is first necessary to understand the involvement of the viral coat proteins with cellular receptors during the normal infection process. There are two adenovirus coat proteins which interact with distinct cellular receptors during the infection process (36). The fiber coat protein, alone, mediates viral attachment to an, as yet, unidentified cellular receptor (14, 22). While adenovirus is known to bind via the fiber to epithelial-derived cells used to propagate adenovirus, the distribution of the adenovirus fiber receptor in tissues targeted for gene therapy has not been established.

Unlike the fiber receptor, the identity and tissue distribution of the penton base receptor are generally known (36). Following fiber-mediated attachment to cells, the penton base binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors which mediate virus internalization into cells via receptor-mediated endocytosis (21, 32, 36). The α_v integrins are cell adhesion receptors expressed by a number of cell types, with the exception of unstimulated hematopoietic cells (13). These receptors bind matrix proteins, including fibronectin and vitronectin, via the amino acid motif, RGD, present in many matrix proteins (6, 13, 29). The penton base, likewise, binds α_v integrins via an RGD sequence (2, 18, 36). The penton base of adenovirus type 5 (Ad5) plays no role in virus attachment (36). The lack of involvement of the penton base in the initial binding event is likely due to the steric hindrance imposed by the long fiber protein and/or the over-10-fold-higher affinity of the fiber-fiber receptor interaction compared with that of the penton-α_v integrin interaction (36).

Many tissues including, endothelial (17), lung epithelial (25), liver (15), muscle (23), and neural (16) tissues have been shown to be transduced by adenovirus. However, the role of the adenovirus fiber protein in the relative transduction efficiencies of these tissues has not been established. In this study we demonstrate that the low level of fiber-mediated, adenovirus binding is a primary factor that limits the transduction efficiency of endothelial and smooth muscle cells relative to epithelial-derived cell lines. The low adenovirus binding level on these cells is presumably due to their low or absent expression of fiber receptor, despite their known expression of the second adenovirus receptor, α_v integrins (5, 9). We also demonstrate that vector binding to endothelial and smooth muscle cells can be dramatically enhanced by circumventing fiber receptor-mediated binding and targeting the attachment of a modified recombinant adenovirus to these cells via α_v integrins. Targeting adenovirus attachment to these cells is accomplished through the use of a bispecific antibody (bsAb) with primary specificity to α_v integrins and a second specificity to an epitope incorporated into the penton base coat protein (34). With this approach, the steric or affinity restraints imposed on penton base-mediated attachment could be simultaneously overcome through complexing the bsAb to an epitope on the penton. Immunoglobulin G Abs typically have high affinity for

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their epitopes, while the extra length of the bsAb (up to 40 nm) could potentially overcome any steric inhibition imposed by the long length of the fiber protein (29 nm). The penton was chosen for incorporating an Ab epitope because the RGD binding domain can be readily manipulated (34) without impairing the ability to make viable adenovirus vectors (2). By increasing vector binding to smooth muscle and endothelial cells via a bsAb, the transduction efficiencies of these cells are significantly enhanced.

MATERIALS AND METHODS

Viruses and cell lines. A549 human alveolar carcinoma cells, 293 human embryonic kidney cells, primary human venule endothelial cells (HuVEC), and primary human intestinal smooth muscle cells (HISMC) were obtained from the American Type Culture Collection (Rockville, Md.). Primary aortic smooth muscle cells were obtained from Cell Systems (Kirkland, Wash.). A549 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (Gibco, BRL, Grand Island, N.Y.). The primary cells, HuVEC, HISMC, and aortic smooth muscle cells, were maintained in MCDB medium (Gibco BRL) supplemented with 10% fetal bovine serum and bovine pituitary extract (Gibco BRL). Adenovirus with E1 and E3 deletions, AdCMV.Gus, contains the β-glucuronidase gene under the control of a cytomegalovirus promoter. AdCMV.Gus was grown in 293 human embryonic kidney, cells which contain the complementary E1 region for virus growth. The virus was purified from infected cells at 2 days postinfection (p.i.) by 3 freeze-thaw cycles followed by two successive bandings on CsCl gradients. Purified virus was dialyzed into 10 mM Tris–150 mM NaCl (pH 7.8) containing 10 mM $MgCl_2$ and 3% sucrose and frozen at −80°C until required for use. Radiolabeled adenovirus was made by adding 50 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, Ill.) per ml to the medium of infected cells at 20 h p.i. at a multiplicity of infection of 5. The infected cells were then harvested at 60 h p.i., and the virus was purified as described above. The activity of the labeled viruses was approximately 10^4 virus particles per cpm. The titer of the infectious particles, expressed in fluorescence focusing units (FFU), was determined by a fluorescence focusing assay with 293 cells (30).

MAbs. The blocking monoclonal antibody (MAb) for all α_v integrins was obtained from the American Type Culture Collection (33). The blocking MAb to the integrin $\alpha_v\beta_5$, P1F6, was kindly provided by Dean Sheppard (University of California—San Francisco) (33). The non-function-blocking 1B1.3.2 MAb to the α_v subunit of α_v integrins was provided by Sarah Bodary (Genentech, San Francisco, Calif.). The M2 MAb directed against the FLAG peptide, DYKDDDDK, was obtained from Kodak (New Haven, Conn.), and the hemagglutinin (HA) MAb, 12CA5, was directed against the influenza HA peptide, YPYDVPCYA.

Preparation of bsAbs. The bsAb L230:FLAG was prepared by chemically cross-linking the L230 MAb with the M2 FLAG MAb by previously described methods (27). Briefly, each Ab was dialyzed into borate-buffered saline (pH 8.5) and concentrated to greater than 2 mg/ml with Centricon 30 concentrators (Amicon, Beverly, Mass.). Succinimidyl-3-(2-pyridyldithiol)-propionate (SPDP) (Pierce Chemicals, Rockford, Ill.) was dissolved in absolute ethanol to 2 mg/ml, and a fourfold molar excess was added to 1 mg of each Ab. After 30 min at room temperature, the pH of the anti-FLAG was lowered by the addition of 0.1 volume of 1 M sodium acetate (pH 4.5), and 1 mg of solid dithiothreitol was added. After about 5 min at room temperature, the reduced anti-FLAG was passed over a Pharmacia PD10 disposable Sephadex G_{25} column in borate-buffered saline and added to 1 mg of the L230 MAb. Cross-linking was allowed to proceed overnight at room temperature; the protein was concentrated to 0.3 ml and then fractionated on an HR 10/30 Superose 12 column (Pharmacia) in borate-buffered saline. Monomeric immunoglobulin G (approximately 30%) was discarded, and the cross-linked material was pooled. It consisted of about 60 to 70% dimer plus trimer, with the rest being higher-molecular-weight aggregates.

Construction of baculovirus and adenovirus transfer plasmids. The base baculovirus transfer plasmid, pRcPB5(ΔRGD), was constructed as previously described (34). This plasmid encodes a penton base deletion mutant encoding an 8-amino-acid deletion of the RGD α_v integrin binding domain, which is replaced by a unique *Spe*I restriction site. The chimeric FLAG and HA penton genes were created as follows. A single sense oligomer and two partially overlapping, complementary antisense DNA oligomers were synthesized to generate the amino acid sequences TSEAAAHAIRGDTYADYKDDDDKGSS for FLAG and TSEAAAHAIRGDTYPYDVPDYAGSS for HA. The sense oligomer contained a 5′ *Spe*I site. It encoded the TSEAAAHAIRGDTY amino acid sequence shared by both constructs and contained an 18-nucleotide sequence at its 3′ end which overlapped and complemented the final 18 nucleotides in the antisense oligomers. The two different antisense oligomers were used in combination with the single sense primer to construct the FLAG and HA inserts. Both antisense oligomers contained a 5′ *Xba*I site and 18 nucleotides which overlapped and complemented the corresponding nucleotides in the sense oligomer. The sense and antisense oligomers for each insert were mixed and filled in with Klenow fragment. The complete double-stranded inserts were then purified and digested with *Xba*I and *Spe*I for cloning into the *Spe*I site of pRcPB5(ΔRGD). Orientation was assessed by *Spe*I-*Asc*I digests of the clones. Clones in the correct orientation were then sequenced in the region of the insert. The sequences of the sense and antisense primers used to create the FLAG and HA chimeric pentons are as follows: GGACTAGTGAGGCGGCGGCCCACGCCATCCGCGGCGACACCTAC, the sense primer for both constructs; GCTCTAGACCCGGCGTAGTCGGGCACGT CGTAGGGGTAGGTGTCGCCGCGGAT, the HA antisense primer; and GCTC TAGACCCCTTGTCGTCGTCGTCCTTGTAGTCGGCGTAGGTGTCGCCGC GGAT, the FLAG antisense primer. The 18-nucleotide complementary regions in the sense and antisense strands are underlined.

Production of recombinant penton protein with baculovirus. Recombinant penton chimeras were produced by using the baculovirus expression system as previously described (34). Briefly, the recombinant baculovirus used to express the pentons was produced by transfecting Tn*5* insect cells with the linearized baculovirus DNA along with the baculovirus transfer plasmids described above by using standard protocols. The resultant recombinant baculovirus was used to infect Tn*5* cells to produce the recombinant protein. Following infection, the cells were harvested 3 days p.i., and the penton protein was purified by using a POROS HQ anion exchange high-performance liquid chromatography (HPLC) column as previously described (34). The recombinant fiber protein was produced as previously described (36).

Immunoprecipitation of recombinant penton proteins. One million Tn*5* cells for each sample were infected with the baculovirus vectors, producing wild-type penton base, PB:WT, or chimeric FLAG penton base, PB:FLAG. The cells were harvested and pelleted 3 days p.i. The cells were then resuspended in 0.5 ml of phosphate-buffered saline (PBS) containing 10 μg of leupeptin per ml, 10 μg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride and subjected to 3 freeze-thaw cycles and a final clearing by centrifugation at 15,000 × *g* for 15 min. A portion of the lysate was then rocked for 2 h at 4°C with agarose-coupled protein A beads (Sigma, St. Louis, Mo.) that contained prebound FLAG MAb or a control L230 MAb. The agarose complexes were washed three times with PBS and then resuspended in 1× nonreducing running buffer. The samples were boiled for 3 min and then run on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. The resulting gel was then stained with Coomassie blue, destained, and photographed.

Construction of recombinant adenovirus. The adenovirus transfer vector pAd37-59 was created by cloning the *Pme*I-to-*Bam*HI fragment (37 to 59 map units) of Ad5 into the cloning vector pNEB193 (New England Biolabs, Beverly, Mass.). The penton chimera genes from the baculovirus transfer vectors, pAcSG2 PB:FLAG and pAcSG2 PB:HA, were each cloned into the pAd37-59 vector to make the adenovirus transfer vectors pAd37-59 PB:FLAG and pAd37-59 PB:HA, respectively. Recombinant adenovirus vectors were made by first isolating viral DNA from the adenovirus vector AdCMV.Gus. AdCMV.Gus is a derivative of *dl*324 (31) with the β-glucuronidase gene (*gus*) under the control of the cytomegalovirus promoter in the E1 region. Purified vector DNA was isolated by digesting the purified virus overnight in 0.5% SDS–10 mM EDTA–10 mM Tris-buffered saline (pH 7.8) containing 1 mg of proteinase K per ml. The solution was then extracted three times with phenol-chloroform and ethanol precipitated. A portion of the purified DNA was then cut overnight with the restriction enzyme *Xmn*I, which cuts wild-type Ad5 at positions 14561 and 15710 within the Ad5 genome. The DNA was then purified and transfected into 293 cells with either linearized pAd 37-59 PB:FLAG or pAd 37-59 PB:HA DNA with the use of calcium phosphate. The resultant recombinant vectors, AdFLAG and AdHA, were plaque-purified three times before being used to grow high-titer viral stocks. PCR and additional functional analyses were used to verify that each vector contained the proper Ab epitope (described below).

Immunofluorescence assays of adenovirus-infected cells. Adenovirus-infected cells that produced chimeric penton base proteins were detected by a standard fluorescence focusing assay with minor modifications (30). 293 cell monolayers in 6-well polystyrene plates were infected with AdCMV.Gus (referred to as AdWT), AdFLAG, or AdHA at a multiplicity of infection of 0.015. The cells were then incubated at 37°C for 2 days and then fixed with methanol. The cells were preblocked with PBS containing 5% bovine serum albumin and then incubated with a 1:500 dilution of a rabbit polyclonal Ab to the penton base, mouse MAb FLAG to the FLAG peptide, or mouse MAb 12CA5 to the HA peptide. Cells were then washed three times and incubated with a 1:200 dilution of either an anti-mouse or anti-rabbit polyclonal, fluorescein isothiocyanate-labeled Ab (Sigma). The cells were washed three times, visualized, and photographed with a Nikon (Tokyo, Japan) Diaphot 200 inverted microscope.

Transduction assays with recombinant adenovirus vectors. HISMC, HuVEC, or A549 or 293 cells (2×10^5 per well) were seeded onto 24-well plates 1 to 2 days prior to the experiments. In assays evaluating the bsAb dose response in fiber receptor-expressing cells, 293 cells were first incubated for 1 h in the presence or absence of 5 μg of recombinant soluble fiber protein per ml. AdFLAG or AdHA (10^8 FFU/ml) were incubated with 0.1 to 10 μg of bsAb per ml for 45 min at room temperature in DMEM–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Each vector-bsAb sample (20 μl) was then added to the fiber-blocked or unblocked 293 cells and incubated for 1 h at room temperature. The cells were then washed two times with DMEM and cultured in DMEM–5% calf serum for 18 h at 37°C. The medium was then aspirated, and the cells were lysed in 300 μl of 1× reporter lysis buffer–10 mM EDTA (Promega, Madison, Wis.). Glucuronidase activity in the cell lysates was

then assayed with the glucuronidase fluorometric assay kit (Tropix, Bedford, Mass.). In transduction experiments using HuVEC and HISMC, the AdFLAG or AdHA vector (10^8 FFU/ml) was preincubated for 45 min at room temperature with 3 μg of the L230 MAb, the FLAG MAb, or the L230:FLAG bsAb per ml or no Ab. Each sample (20 μl) was then added to HuVEC or HISMC for 1 h at room temperature. The cells were then washed twice and incubated for 18 h at 37°C. Glucuronidase activity was then assayed as described above. In specificity experiments using HuVEC and HISMC, the AdFLAG or AdHA vector (10^8 FFU/ml) was preincubated in the presence or absence of 50 μg of FLAG MAb per ml for 30 min at room temperature. The L230:FLAG bsAb was then added at 3 μg/ml for an additional 30 min at room temperature. Each vector sample (20 μl) was then added to HuVEC or HISMC preblocked in the presence or absence of 50 μg of competing L230 MAb per ml. The cells were incubated for 1 h at room temperature and treated as described above to determine the β-glucuronidase activity.

Binding assays with recombinant adenovirus vectors. Confluent monolayers of 293 cells in 24-well plates were preincubated in the presence or absence of 300 μl of a 5-μg/ml concentration of the recombinant fiber protein for 1 h at 4°C. The [^{3}H]thymidine-labeled (24,000 cpm) AdFLAG or AdWT vector was preincubated with 3 μg of the L230:FLAG bsAb, the FLAG MAb, or the L230 MAb per ml for 1 h at 4°C in a total volume of 0.3 ml. Each sample was added to the fiber-blocked or unblocked 293 cells and incubated for 1 h at 4°C. The cells were then washed three times with PBS and solubilized in 200 μl of a 1% SDS solution, and the radioactivity was determined in a scintillation counter.

RESULTS

Adenovirus binds poorly to smooth muscle and endothelial cells relative to A549 cells. Although many tissues have been reported to be transduced by adenovirus, the relative transduction efficiencies of different tissues compared with those of the host epithelial cells are not generally known. Smooth muscle cells and endothelial cells are clinically relevant targets for gene therapy in order to prevent restenosis of angioplastied arteries and to promote or to prevent angiogenesis (19). Even though these cell types have been previously reported to be transducible by adenovirus (17, 26), direct comparison of smooth muscle and endothelial cells to epithelial-derived cells showed a significant reduction in transduction efficiency by adenovirus.

The relative transduction efficiencies of primary HISMC and primary HuVEC were compared with the transduction efficiency of A549 alveolar carcinoma cells by using a recombinant adenovirus vector (Fig. 1A). The recombinant vector, AdCMV .Gus, contains the β-glucuronidase gene in the deleted E1 region under control of a cytomegalovirus promoter. Although gene delivery to smooth muscle and endothelial cells did occur, the relative expression levels were 690- and 130-fold lower, respectively, than the expression levels in A549 cells. To assess the role of the fiber receptor in transduction efficiencies of the cells, the relative levels of total and fiber-specific Ad5 binding in the three cell lines were determined (Fig. 1b). A549 cells bound 30- and 10-fold-larger amounts of labeled Ad5 than did smooth muscle and endothelial cells, respectively. Furthermore, while over 95% of the A549 binding was inhibitable by 5 μg of the recombinant fiber protein per ml, the binding of Ad5 to HISMC was unaffected by competing fiber protein.

Targeting adenovirus binding to α_v integrins with bsAbs. It was hypothesized that by increasing vector binding to HISMC and HuVEC, their transduction efficiencies could be simultaneously increased. One way of increasing vector binding to these cells was through the use of bsAbs (27). A bsAb was constructed by chemically linking the M2 FLAG MAb with the L230 MAb (Fig. 2). The FLAG MAb binds the FLAG polypeptide, DYKDDDDK, while the L230 MAb binds α_v integrins. The L230 MAb was chosen because smooth muscle and endothelial cells express α_v integrins and because adenovirus normally uses α_v integrins to enter cells (5, 9, 36). Incorporation of the FLAG peptide into the penton base protein would thereby allow recognition of the adenovirus vector by the L230:FLAG bsAb.

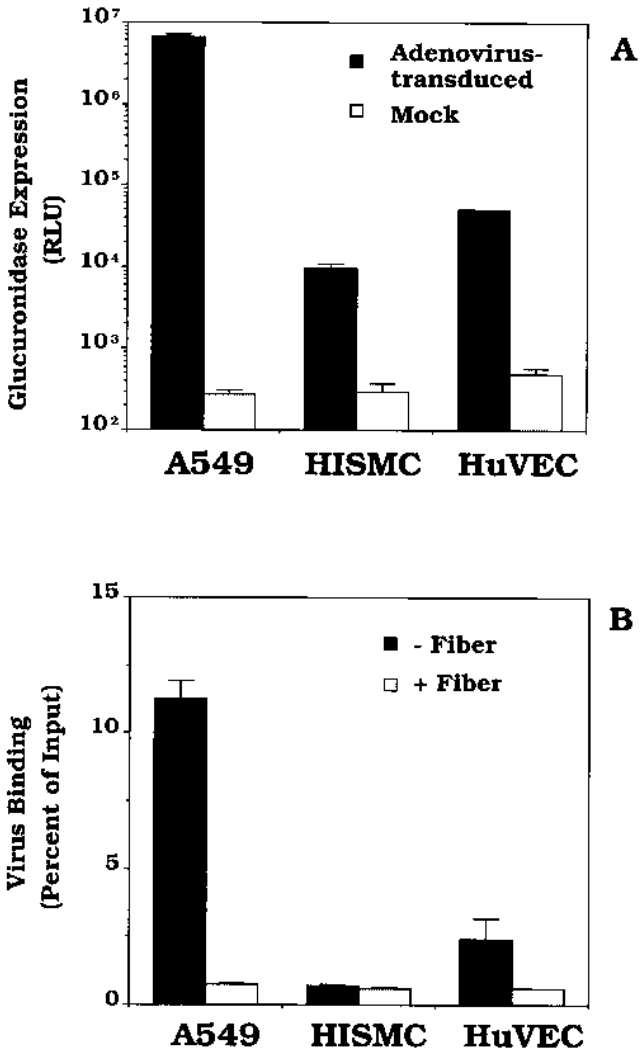

FIG. 1. Direct correlation between adenovirus-mediated cell transduction and binding. (A) Adenovirus-mediated transduction of A549, primary endothelial, and primary smooth muscle cells. Confluent monolayers of A549 carcinoma cells, primary HuVEC, or primary HISMC in 35-mm wells were incubated for 1 h in the absence (mock) or presence of 5×10^6 FFU of AdCMV.Gus vector at 37°C in DMEM culture medium. Cells were then washed three times with PBS and further incubated for 1 day at 37°C. Glucuronidase activity was determined as described in Materials and Methods. The results are the averages of duplicate measurements. (B) Fiber-specific adenovirus binding to epithelial, endothelial, and smooth muscle cells. One million A549 carcinoma cells, primary HuVEC, or primary HISMC were preincubated for 1 h in the absence or presence of 5 μg of recombinant soluble fiber protein per ml. [^{3}H]thymidine-labeled (24,000 cpm) adenovirus was then added to each well and incubated for 1 h at 4°C in a total volume of 0.3 ml of culture medium. Cells were then washed three times, and the total cell-associated counts were measured in duplicate in a scintillation counter.

Characterization of recombinant FLAG penton. A baculovirus transfer plasmid containing the penton base gene with an 8-amino-acid deletion of the RGD integrin-binding domain was used to create the penton base-FLAG chimera (PB:FLAG). In this plasmid the RGD deletion is replaced by an *Spe*I restriction cloning site. The PB:FLAG chimeric gene was created by cloning into the penton plasmid an insert containing the original RGD coding sequence juxtaposed to the FLAG peptide coding sequence (Table 1) (34). Additional spacer amino acids were also incorporated into the penton insert to assure availability of the FLAG epitope to the FLAG MAb. A

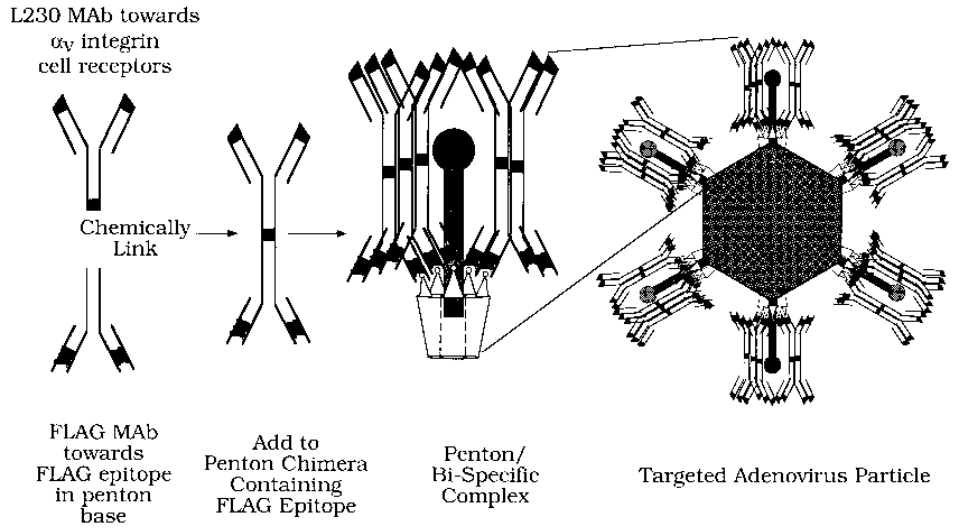

FIG. 2. Targeting adenovirus binding to α_v integrins with bsAbs. The L230 MAb directed against α_v integrins is chemically linked to the FLAG MAb directed against the FLAG peptide epitope as described in Materials and Methods. The purified bsAb is then added to purified recombinant adenovirus containing the FLAG peptide epitope incorporated into the receptor binding domain in the penton base. The resulting bsAb-adenovirus complex imparts the virus with high-affinity binding to α_v integrin cellular receptors. The dimensions are shown approximately to scale: antibody, approximately 20 nm long; fiber protein, 29 nm long.

similar penton chimera incorporating the HA peptide was also cloned into the baculovirus transfer plasmid to create the chimera PB:HA. This penton chimera served as a control in these studies.

Recombinant PB:FLAG protein was produced by using the baculovirus expression system in Tn*5* cells. The recombinant protein was first immunoprecipitated to determine whether PB:FLAG was recognized by the FLAG MAb and was the correct size (Fig. 3). The FLAG MAb immunoprecipitated the PB:FLAG that was just slightly larger than the purified wild-type penton base standard because of the 18 additional amino acids present in the PB:FLAG chimera. The FLAG MAb did not precipitate PB:WT, and the control L230 MAb did not precipitate either PB:FLAG or PB:WT. Cell adhesion studies performed to assess the functionality of the RGD sequences in PB:FLAG and PB:HA showed that both penton chimeras could mediate cell adhesion in an $\alpha_v\beta_3$- and $\alpha_v\beta_5$-dependent manner as previously shown for PB:WT (data not shown) (36).

Construction of recombinant adenovirus containing the FLAG penton chimera. After the functionality of the recombinant FLAG penton was demonstrated, the FLAG penton gene was then used to construct a new adenovirus vector, AdFLAG, which contained the chimeric FLAG penton protein. AdFLAG was derived directly from the vector AdCMV-.Gus and is identical to it except for the penton base gene. A similar vector, AdHA, containing the HA epitope in the penton base gene was also constructed. Immunofluorescence detection of AdFLAG- or AdHA-infected 293 cells was done to verify that AdFLAG contained the functional FLAG epitope (Fig. 4). Cells infected with either the AdFLAG or the base adenovirus vector lacking the FLAG epitope (AdWT) were both detected by immunofluorescence with the use of a polyclonal Ab against the penton base. However, only cells infected with AdFLAG were detected with the FLAG MAb. The similar vector, AdHA, was specifically recognized by the HA MAb but not the FLAG MAb. These results demonstrate that the FLAG MAb recognizes the virus-derived penton chimera.

Transduction and binding of fiber receptor-blocked 293 cells by using AdFLAG complexed with L230:FLAG bsAb. Further studies were performed to determine whether the L230:FLAG bsAb complexed to the AdFLAG vector can mediate transduction of fiber receptor-containing cells via α_v integrins. Cell transduction by the AdFLAG-bsAb complex was then determined in the presence or absence of competing soluble fiber protein (Fig. 5). AdFLAG or a control vector, AdHA, was incubated with increasing concentrations of L230:FLAG bsAb. The virus-antibody solutions were then incubated with fiber receptor-containing 293 cells that had been preincubated in medium with or without soluble competing fiber protein. In the absence of any bsAb, the soluble fiber protein blocked over 95% of the transduction of both the AdHA and AdFLAG vectors (Fig. 5). In the presence of fiber, increasing concentrations of L230:FLAG did not increase AdHA-mediated β-glucuronidase transduction (Fig. 5B). However, increasing concentrations of L230:FLAG added to AdFLAG significantly increased β-glucuronidase transduction of the fiber-blocked cells in a dose-dependent manner (Fig. 5A). Furthermore, L230:FLAG was able to completely restore transduction to the same levels as in the absence of competing fiber. Half of the original transduction in the absence of fiber was restored in the presence of fiber at 0.1 μg of bsAb per ml. Under the conditions of the experiment (10^8 FFU/ml, 100 AdFLAG particles

TABLE 1. Antibody recognition motifs incorporated into penton chimeras[a]

Ab	Sequence
PB:WT	**N D**– – – – – – **H A I R G D T Y** – – – – – – – – – – – – **R A**
PB:FLAG	**N D** T S E A A A **H A I R G D T Y** A D Y K D D D D K G S S **R A**
PB:HA	**N D** T S E A A A **H A I R G D T Y** P Y D V P D Y A – G S S **R A**

[a] Boldface amino acids represent the wild-type penton base sequence; underlined amino acids represent motifs recognized by the anti-FLAG or anti-HA MAbs.

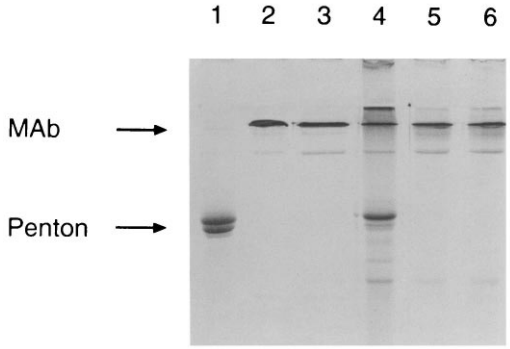

FIG. 3. Immunoprecipitation of PB:FLAG but not PB:WT with FLAG antibody. Samples of 10^6 Tn5 cells infected 3 days prior with the PB:WT or PB:FLAG baculovirus vectors were pelleted, resuspended in 0.5 ml of PBS plus protease inhibitors, and freeze-thawed three times. The samples were cleared by centrifugation and then incubated for 2 h at 4°C with protein-A agarose beads containing approximately 5 μg of prebound FLAG MAb or control L230 MAb. The beads were then washed three times with PBS, and 40 μl of 1× Laemmli buffer was added to each sample. The samples were boiled for 3 min, and then 20 μl of each sample was run on a 10% polyacrylamide–0.5% SDS gel. The gel was stained with Coomassie blue, destained, and then photographed. Lanes: 1, 5 μg of HPLC-purified PB:WT standard; 2, 5 μg of purified FLAG MAb; 3, FLAG MAb plus PB:WT Tn5 cell lysate; 4, FLAG MAb plus PB:FLAG Tn5 cell lysate; 5, L230 MAb plus PB:WT; 6, L230 MAb plus PB:FLAG.

per FFU, and 60 FLAG epitopes per AdFLAG particle), this concentration of bsAb represents an approximately 1:3 ratio of bsAb molecules to the available FLAG epitopes, or 20 bispecific antibodies per virus particle. At 0.3 μg of bsAb per ml, the block by fiber is completely restored, corresponding to 60 bsAb molecules per virus particle. These results strongly suggest that the AdFLAG vector when complexed with L230:FLAG can mediate transduction of cells via α_v integrins. Furthermore, they demonstrate that there is no loss in L230:FLAG-mediated transduction efficiency relative to the efficiency of purely fiber-mediated adenovirus transduction of cells.

To provide further evidence that the increased transduction efficiency by the AdFLAG-bsAb complex was due to increased binding, radiolabeled vector binding to 293 cells in the presence or absence of the L230:FLAG bsAb was determined (Fig. 6). Radiolabeled AdFLAG or its parental vector, AdCMV.Gus (AdWT), was preincubated with the L230:FLAG bsAb, control FLAG MAb, or control L230 MAb. The vector-antibody complexes were then added to 293 cells which had been preincubated in the presence or absence of competing fiber. AdWT binding to the fiber-treated cells was blocked regardless of Ab (Fig. 6A). In contrast, fiber did not block the binding of the AdFLAG vector preincubated with the L230:FLAG bsAb. Neither the FLAG MAb nor the L230 MAb alone was able to overcome the block. Furthermore, the binding level achieved by the AdFLAG-bsAb complex was the same as the binding level in the absence of competing fiber protein (Fig. 6B). These findings demonstrate that the increase in transduction efficiency of the AdFLAG-bsAb complex results from increased vector binding. In addition, these results show that the AdFLAG-bsAb complex is just as efficient in binding to fiber-blocked cells as the vector alone in binding via the fiber.

The AdFLAG-L230:FLAG complex transduces cells lacking fiber receptor more efficiently than does vector alone. Further experiments were performed to determine whether the L230:FLAG bsAb complexed to the AdFLAG vector can increase transduction of endothelial and smooth muscles cells which lack high levels of fiber-mediated virus binding (Fig. 7). Cell

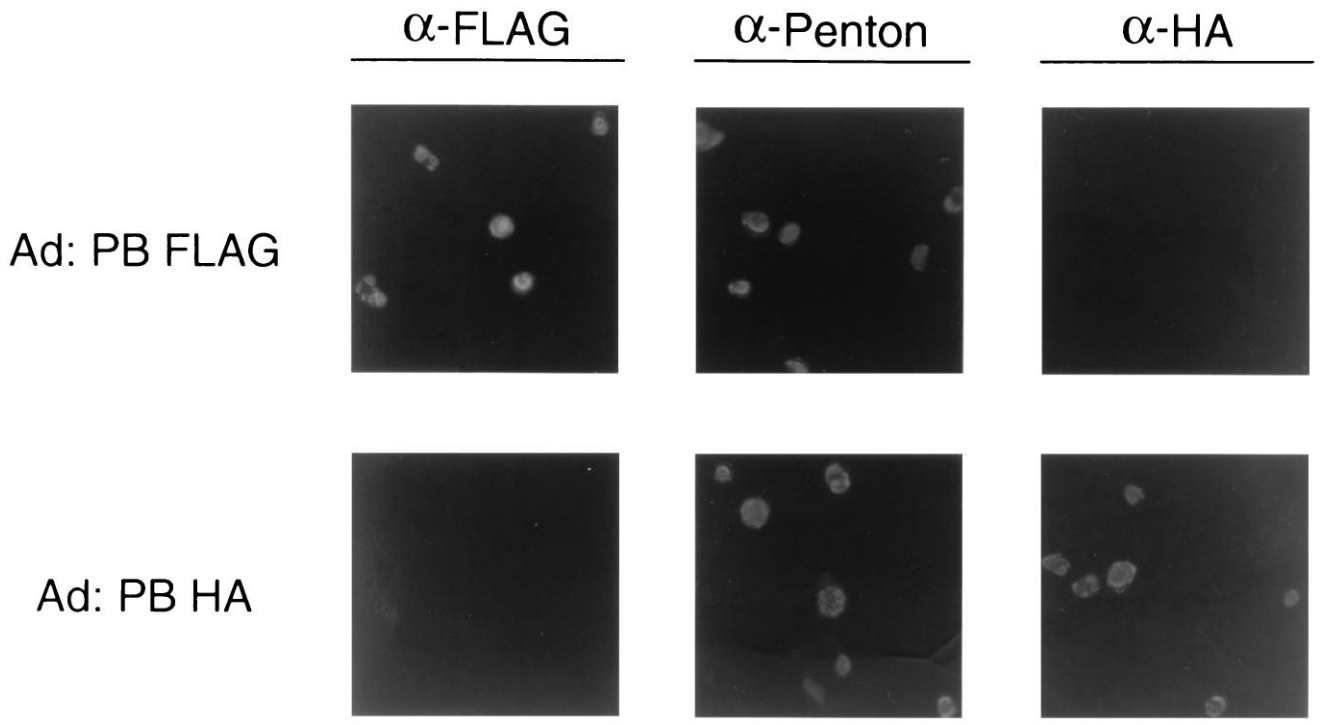

FIG. 4. Immunofluorescence of AdFLAG and AdHA virus. 293 cells were plated at 50% confluency in 6-well plates 1 day prior to infection with AdFLAG or AdHA vectors at a multiplicity of infection of 0.01. After 2 days of incubation at 37°C, the cells were washed, fixed, and preblocked as described in Materials and Methods. The cells were probed with a polyclonal Ab against the penton base, the FLAG MAb, or the HA MAb. The cells were then washed and probed with the appropriate fluorescein isothiocyanate-labeled anti-mouse or anti-rabbit polyclonal Abs.

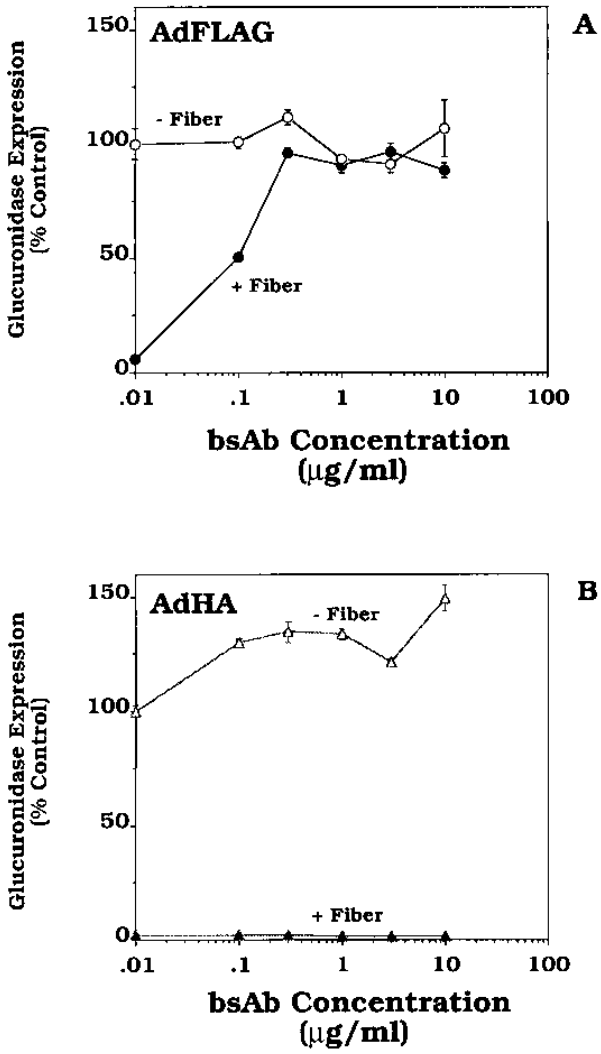

FIG. 5. Dose response of β-glucuronidase expression by targeting α_v integrins on 293 cells blocked with soluble fiber. Confluent monolayers of 293 cells in 24-well plates were preincubated in the absence or presence of 300 μl of 5-μg/ml solution of Ad5 fiber protein for 1 h at room temperature. The AdFLAG (A) or AdHA (B) adenovirus vector (10^8 FFU/ml) was preincubated with the indicated concentrations of the L230:FLAG bsAb for 45 min at room temperature in DMEM–20 mM HEPES. Each vector sample (20 μl) was then added to the fiber-blocked or unblocked 293 cells and incubated for 1 h at room temperature. The cells were then washed twice with DMEM and incubated for 18 h at 37°C in DMEM–5% calf serum. The medium was then aspirated, and the cells were lysed in 300 μl of 1× lysis buffer containing 10 mM EDTA. Each sample (3 μl) was then assayed fluorometrically with a luminometer. The results are the averages for duplicate samples.

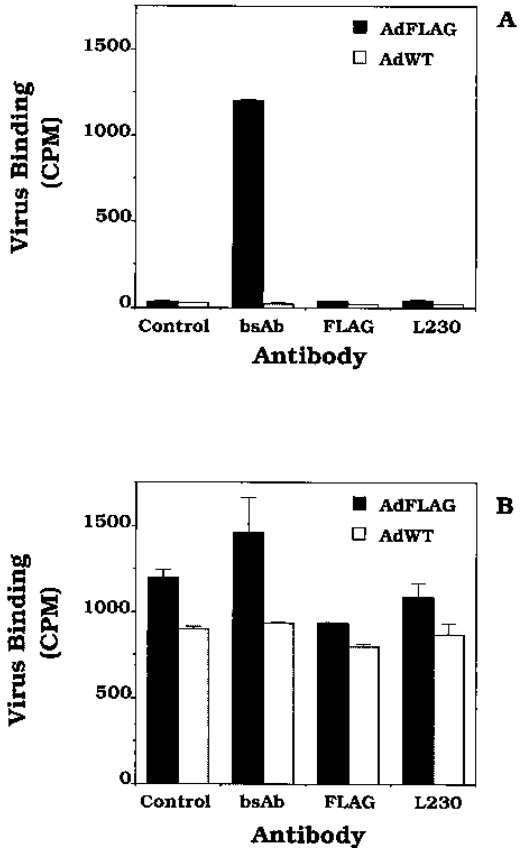

FIG. 6. Increased binding of radiolabeled AdFLAG complexed with bsAb to 293 cells. Confluent monolayers of 293 cells in 24-well plates were preincubated in the presence (A) or absence (B) of 300 μl of a 5-μg/ml solution of Ad5 fiber protein for 1 h at 4°C. [^{3}H]thymidine-labeled (24,000 cpm) AdFLAG or AdWT vector was preincubated with 3 μg of the L230:FLAG bsAb, the FLAG MAb alone, or the L230 MAb alone per ml for 45 min at 4°C in a total volume of 20 μl. Each sample was then added to the fiber-blocked or unblocked 293 cells and incubated for 1 h at 4°C. The cells were then washed three times with PBS. The cells were then solubilized in a 1% SDS solution and counted in a scintillation counter. The reported results are the averages for duplicate samples.

transduction by either the AdFLAG or AdHA vector was determined following preincubation with the L230:FLAG bsAb, the FLAG MAb, or the L230 MAb. Neither the L230:FLAG, FLAG, or L230 Abs caused any significant increase in transduction by the AdHA vector. However, the L230:FLAG bsAb, but not the FLAG or L230 MAb alone, significantly increased the transduction of smooth muscle (Fig. 7A) and endothelial (Fig. 7B) cells by the AdFLAG vector. Transduction of the endothelial and smooth muscle cells by the AdFLAG-bsAb complex was increased nine- and sevenfold, respectively, compared with that with the AdFLAG vector alone.

The specificity of transduction by the AdFLAG-bsAb complex was evaluated by preincubation of the cells with L230 MAb or preincubation of the virus with FLAG MAb (Table 2). Any resulting block in transduction would show that the increased transduction by the AdFLAG-bsAb complex was specific to the dual interaction of the L230:FLAG bsAb with the FLAG epitope on the virus and with α_v integrins on the cells. Preincubation of the control vector, AdHA, with FLAG MAb prior to its incubation with the L230:FLAG bsAb caused no significant reductions in transduction by AdHA in either HISMC or HuVEC. However, preincubation of AdFLAG with FLAG MAb prior to incubation with L230:FLAG bsAb blocked the increased transduction by the AdFLAG-bsAb complex alone in both the endothelial and smooth muscle cells. Likewise, preincubation of the cells with the L230 MAb blocked transduction of the cells by the AdFLAG-bsAb complex but had no significant effect on the transduction by AdHA preincubated with the L230:FLAG bsAb. These results demonstrate that the dual interactions of the L230:FLAG bsAb with AdFLAG and with α_v integrin cell receptors are necessary to achieve the increase in transduction of HISMC and HuVEC.

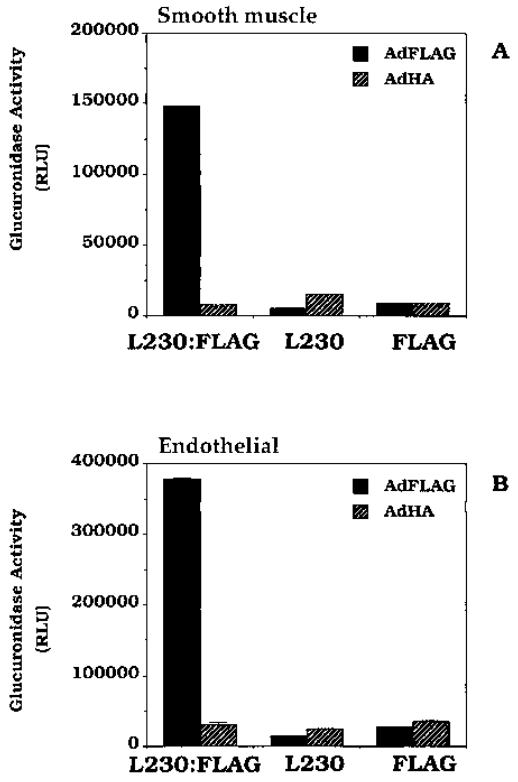

FIG. 7. L230:FLAG bsAb increases transduction of HuVEC (A) and HISMC (B). HuVEC or HISMC were plated in 24-well plates 2 days prior to use. AdFLAG or AdHA vectors (10^8 FFU/ml) were preincubated with 3 μg of L230:FLAG bsAb, L230 MAb, or FLAG MAb per ml or no Ab for 45 min at room temperature. Each vector sample (20 μl) was then added to the HuVEC or HISMC and incubated for 1 h at 37°C. The cells were then washed twice with DMEM and then further incubated at 37°C for 18 h in DMEM–10% fetal bovine serum. The cells were then aspirated and assayed for β-glucuronidase activity as described in the legend to Fig. 5. RLU, relative light units.

DISCUSSION

A general paradigm in gene therapy is that relative to other vector systems virtually all cells are efficiently transduced by adenovirus. Similar to previous reports, we have been able to demonstrate the transduction of endothelial and smooth muscle cells by adenovirus (17, 26). However, we have shown that these two cell types, which are prime targets for gene therapy, lack significant levels of fiber-mediated vector binding (Fig. 1). Their low levels of binding are directly correlated with their poor transduction efficiencies compared with those of A549 and other epithelial-derived cell lines which show comparatively high levels of fiber-mediated adenovirus binding. It is also likely that other tissues targeted for adenovirus-mediated gene therapy may likewise lack high expression levels of the fiber receptor. The significant increases in binding and transduction of cells lacking the high fiber receptor expression levels shown here demonstrate that bsAbs can be successfully used to expand the range of tissues amenable to efficient adenovirus-mediated gene therapy.

Smooth muscle cells are targets for gene therapy to prevent their proliferation and resultant obstruction of coronary arteries following balloon angioplasty (19). While data for primary intestinal smooth muscle is presented here, similarly, we have found undetectable levels of the fiber receptor as determined by fiber-specific adenovirus binding in primary human aorta smooth muscle cells. These cells are, likewise, more efficiently transduced with the AdFLAG-bsAb complex than with an unmodified vector.

The α_v integrins are promising tissue-specific receptors for targeted gene therapy. α_v integrin expression is activated in a majority of melanomas (1) and glioblastomas (12). Targeting therapeutic adenovirus to the α_v integrins on these cells would allow delivery of a toxic gene, for example, while avoiding gene delivery to healthy, surrounding tissue. Furthermore, the integrin $\alpha_v\beta_3$ is expressed on proliferating endothelial cells (3, 4). Targeting the $\alpha_v\beta_3$ receptor on these cells may be useful in preventing their proliferation, such as in tumor growth or retinal disease, or to promote further vascularization, such as the revascularization of ischemic tissue.

bsAb-mediated transduction with adenovirus demonstrates the feasibility of targeting vectors to specific tissues via tissue-specific receptors. However, potential drawbacks of this system may include complement activation, clearance by Fc receptors, virus-antibody aggregation, and immune responses against the Ab components. Despite these potential drawbacks of the present system, they can all presumably be overcome. For example, a fusion protein comprised of a single-chain Ab directed towards adenovirus and a second single-chain Ab directed towards a targeted cellular receptor would avoid Fc receptor recognition and any virus-antibody aggregation problems. Furthermore, the use of human or humanized Abs will likely prevent immune responses against the Abs (although immune responses against the viral coat proteins are probably of greater concern).

Targeting gene delivery via the penton base also demonstrates that attachment via the fiber, per se, is not required for the efficient transduction of cells by adenovirus. Transduction of fiber-blocked 293 cells by using the vector-antibody complex was just as efficient as the fiber-mediated transduction of the

TABLE 2. Specificity of glucuronidase transduction by AdFLAG-L230:FLAG bsAb complex

Condition	Glucuronidase activity in[a]:			
	Smooth muscle cells		Endothelial cells	
	AdFLAG	AdHA	AdFLAG	AdHA
Complex added directly to unblocked cells[b]	148.1 (0.3)	7.8 (0.1)	378.2 (1.6)	31.5 (1.5)
FLAG MAb added to vector prior to complex formation[c]	8.4 (0.1)	9.2 (0.1)	27.5 (0.7)	35.5 (0.7)
L230 MAb added to cells prior to complex addition[d]	5.3 (0.1)	15.5 (0.1)	14.6 (0.5)	24.2 (2.1)

[a] HuVEC or HISMC were plated in 24-well plates 2 days prior to transduction. For details, see Materials and Methods. AdFLAG or AdHA vectors (10^8 FFU/ml) were incubated with cells for 1 h. The cells were then washed and incubated at 37°C for 18 h. Glucuronidase activity was determined; the results, expressed as relative light units (10^3), are the averages for duplicate measurements. Standard errors are given in parentheses.

[b] AdFLAG or AdHA was preincubated with 3 μg L230:FLAG bsAb 45 min prior to addition to the cells.

[c] AdFLAG or AdHA was preincubated in the presence or absence of 50 μg FLAG MAb per ml prior to the addition of 3 μg of the L230:FLAG bsAb per ml.

[d] Cells were preincubated for 45 min with 50 μg of L230 MAb per ml prior to the addition of the complex as described in footnote *b*.

uncomplexed vector. Therefore, in the absence of fiber-mediated binding, the transducing activity of an adenovirus particle is not compromised by attachment through the bsAb. Although many tissues may lack fiber receptor expression, a number of tissues are known to express the fiber receptor, including certain lymphocyte cell lines (28), melanoma cells (35), and many epithelial-derived cell lines (10, 22). Therefore, the efficient targeting and limitation of transduction to a particular cell or tissue will necessitate adenovirus vectors which lack fiber receptor-binding activity. Such vectors may likewise require special receptor-expressing cell lines in order to propagate them. In any case, it will be important to determine the levels of adenovirus receptors in tissues targeted for gene therapy. If the level of fiber receptor expression is low in a given tissue, using bsAbs to target adenovirus to receptors that are expressed by the tissue is likely to increase the efficiency and specificity of gene transfer.

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