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Development of adenovirus serotype 35 as a gene transfer vector

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Abstract

While 51 human adenoviral serotypes have been identified to date, the vast majority of adenoviral vectors designed for gene transfer have been generated in the adenovirus serotype 5 (Ad5) backbone. Viral infections caused by Ad5 are endemic in most human populations and the majority of humans carry preexisting humoral and/or cellular immunity to Ad5 which may severely limit the use of Ad5-based vectors for gene therapy applications. To circumvent this preexisting Ad5 immunity, we have identified Ad35 as an alternative adenoviral serotype to which the majority of humans do not have neutralizing antibodies. Importantly, Ad35 can be grown to high titers with a low particle-to-PFU ratio. As a prerequisite for the development of Ad35 for use as a gene transfer vector, a genome organization map was constructed using the available Ad35 sequence information, and E1a-deficient Ad35 vectors encoding marker genes were generated. Ad35 biodistribution in mice was assessed following intravenous administration and compared with that of Ad5. Extremely low levels of Ad35 were detected in all organs evaluated, including liver, lung, spleen, and bone marrow, while Ad5 displayed high transduction of these organs. Due to the lack of Ad35 liver tropism, minimal hepatotoxicity was observed in mice treated with Ad35. Furthermore, the half-life of Ad35 in mouse blood was found to be two to three times longer than that of Ad5. These data suggest that either mice do not express the Ad35 cell surface receptor or that Ad35 does not efficiently transduce mouse cells in vivo following systemic delivery. Therefore, to begin to elucidate the Ad35 cell entry mechanisms, in vitro competition studies were performed. These data demonstrated that Ad35 cell entry is CAR independent, and may involve protein(s) expressed on most human cells.

Keywords: Alternative adenovirus serotype; Gene delivery; Preexisting immunity

Introduction

Several attractive features of adenoviruses make them suitable candidates for development as gene transfer vectors. Some of these features include: well-studied biology, growth to high titers, and extremely high efficiency in delivery of therapeutic genes to both dividing and nondividing cells in vivo and in vitro. Although 51 human adenoviral serotypes have been recognized to date, most of the currently used human adenoviral vectors for gene transfer are based on the subgroup C adenoviral serotypes 2 and 5. The primary reason for the selection of these two serotypes for development as vectors is their well-characterized genomes and favorable growth characteristics. However, preclinical and clinical studies with these vectors exposed several limitations associated with their use in human gene therapy. One major limitation is the presence of preexisting immune responses in humans. Viral infections due to subgroup C adenoviruses are endemic in most human populations. Antibodies specific to adenovirus were detected in 97% of individuals and more than 50% of people carry preexisting humoral and cellular immune responses to Ad2 and Ad5 (Chirmule et al., 1999). Preexisting antibodies can neutralize the vector and may block vector uptake and transduction in vivo (Smith et al., 1996). Furthermore, adenoviral vector preimmunization in mice was demonstrated to significantly increase vector-mediated liver toxicity on reexposure (Vlachaki et al., 2002), and the presence of anti-adenovirus

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antibodies in human blood may activate the complement system and induce inflammatory reactions in the presence of adenovirus (Cichon et al., 2001). Thus, preexisting immunity to Ad5 may severely limit the use of these vectors for gene therapy, especially for systemic delivery applications.

Several approaches have been employed to circumvent this preexisting immunity. Some of the approaches included transient immune suppression at the time of vector delivery (Smith et al., 1996), induction of tolerance to adenoviral capsid proteins (Yang et al., 1999; Ilan et al., 1998), plasmapheresis to remove adenovirus-specific antibodies (Rahman et al., 2001), coating of adenoviruses with polymers (Fisher et al., 2001; Croyle et al., 2002), development of animal adenoviruses, such as bovine and porcine, as vectors (Reddy et al., 1999a,b), and use of chimeric vectors in which proteins carrying neutralization epitopes are swapped with those from rare adenoviral serotypes (Roy et al., 1998; Miyazawa et al., 1999). Each of these approaches had limited success in rodent models, and the utility of these methods for treatment of humans remains unclear.

An alternative strategy, which is receiving increasing attention, is the development of vectors derived from alternative human adenoviral serotypes which are underrepresented in the human population. Indeed, administration of an Ad2-based vector in Ad5 immunized mice resulted in efficient transduction of mouse tissues, demonstrating the utility of this strategy (Mastrangeli et al., 1996, Mack et al., 1997). However, both Ad2 and Ad5 are closely related serotypes from subgroup C. Historically, adenovirus has been classified into six major subgroups, A–F, according to their oncogenic and hemagglutination properties and their DNA homology (reviewed by Shenk, 1996). Subgroup C adenoviruses have been used predominately for the generation of gene transfer vehicles due to their favorable growth properties, broad tropism, and well-studied genome. To date, few gene therapy vectors have been generated from adenoviruses from subgroups other than C (Abrahamsen et al., 1997).

In this work, we screened 200 human serum samples for adenovirus neutralization, and identified Ad35 as a serotype that is underrepresented in the human population. Serotype 35 belongs to subgroup B and was first identified in 1975 from the lungs and kidneys of a renal transplant patient with a fatal infection (Myerowitz et al., 1975). More recently, Ad35 was isolated from urine samples or urinary tracts of renal and bone marrow transplant recipients and immunocompromised AIDS patients (Flomenberg et al., 1987). Based on the DNA homology, the members of subgroup B are divided into two clusters, named B1 and B2 (Wadell, 1984). The B1 cluster is characterized by tropism for respiratory epithelium and includes Ad3, 7, 16, and 21. The B2 cluster displays tropism for the urinary tract, and includes serotypes Ad11, 14, 34, and 35 (Wadell, 1984). Basler and colleagues (Basler et al., 1996; Basler and Horwitz, 1996) reported the nucleotide sequence and transcription map for the early region 3 (E3) of Ad35, while, more recently, the complete nucleotide sequence of the Ad35 genome has been determined (GenBank Accession No. X049983). However, to date, a genome organization map of Ad35 has not been available.

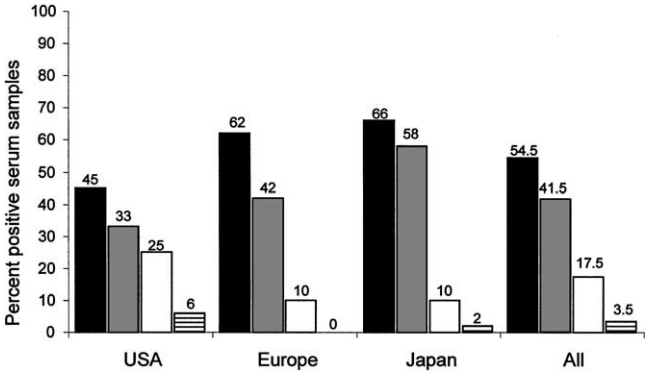

Fig. 1. Ad5 and Ad35 neutralizing antibodies in human serum. A total of 200 human serum samples collected from the United States, Europe, and Japan were screened for the presence of Ad5 and Ad35 neutralizing antibodies. Neutralizing antibody titers were scored as the reciprocal of the highest dilution of serum that prevented CPE formation. Black bars represent percentages of samples with detectable antibodies to Ad5, and gray bars represent percentages of samples with anti-Ad5 titers ≥8. Open bars represent percentages of serum samples with detectable anti-Ad35 antibodies, and striped bars represent percentages of serum samples with anti-Ad35 antibody titers ≥8. USA, Europe, and Japan indicate samples collected from these locations. "All" represents a compilation of antibody titer data from all samples. The numbers above the bars indicate the percentages of samples with a positive antibody titer.

This report describes the development of Ad35 into early-generation, E1a- and E1-deficient gene transfer vectors. Ad35 biodistribution, hepatotoxicity, and blood clearance rate in mice following systemic administration were evaluated. The relative Ad35 transduction efficiency of several human cell lines and two mouse cell lines was assessed and compared with that of an Ad5 vector. Finally, Ad35 in vitro growth characteristics and cell entry mechanisms were evaluated.

Results

Human serum screening and Ad35 genome organization

Ad35 had been previously identified as a serotype that is rare in the human population (Flomenberg et al., 1987). To verify these data, a total of 200 human serum samples collected from the United States, Europe, and Japan were tested for the presence of neutralizing antibodies against Ad5 and Ad35 (Fig. 1). As expected, the majority of human serum samples, 109, contained neutralizing antibodies to Ad5, indicating widespread prevalence of Ad5 exposure. Interestingly, a very high percentage (41%) of serum samples had titers ≥1:8 against Ad5. In contrast, 17.5% of the serum samples tested positive for neutralizing antibodies against Ad35, and most of these samples displayed low

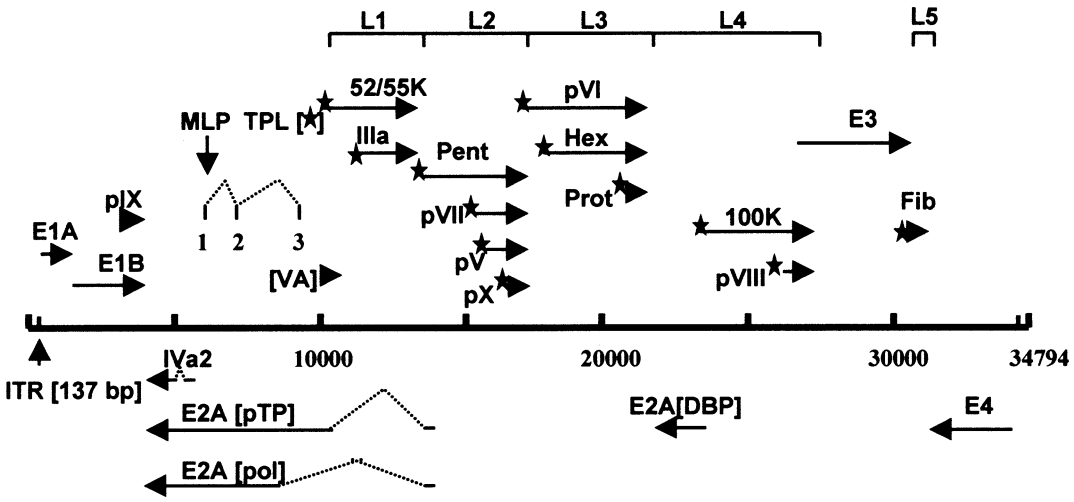

Fig. 2. Ad35 genome organization. The Ad35 genome organization map is based on sequence homologies to other adenovirus serotypes. The central solid line represents the Ad35 genome (34,794 bp) and arrows below and above the line represent mRNA from the right (r) and left (l) strands, respectively. Solid lines indicate the sequences present in mature mRNA, dotted lines represent introns, and arrowheads indicate poly(A) sites and direction of transcription. The locations of early region genes (E), late region genes (L), MLP (major late promoter), TPL (tripartite leader represented by stars), and VA (virion-associated) RNA genes are displayed. The locations of genes within the early and late regions are also identified. The locations of genes coding for hexon (hex), protease (pro), and fiber (fib) are also displayed.

antibody titers. Interestingly, only 3.5% of serum samples displayed Ad35 antibody titers $\geq$1:8. A similar trend was displayed in the samples from all three locations (Fig. 1). These data suggested that Ad35 was a promising serotype for vector development.

While the complete nucleotide sequence of the Ad35 genome is available (GenBank Accession No. AX049983), the genome organization had not been published. Therefore, as the first step toward Ad35 vector development, we generated a genome organization map by comparison to the genome of Ad5 and using conserved promoter sequences, cap sites, splice donor and splice acceptor sites, and poly(A) signal sites (Fig. 2). We performed a BLAST search of the GenBank database using the translated amino acid sequences for the predicted Ad35 open reading frames of at least 70 amino acids. While the Ad5 genome is 35,935 bp with a G + C content of 55.2%, the Ad35 genome is 34,794 bp with a G + C content of 48.9%. The genome of Ad35 is flanked by 137-bp inverted terminal repeats (ITRs), which are longer than those of Ad5 (103 bp). The packaging signal of Ad35 is located at the left end of the genome with five putative "A" repeats. The locations of all the Ad35 early regions, E1A, E1B, E2A, E2B, E3, and E4, are similar to those in other human adenoviruses for which the complete nucleotide sequence is available. Similarly, the sites of the intermediate region genes encoding pIX and IVa2 in the genome of Ad35 are also conserved. The late transcription unit of Ad35 is transcribed from the major late promoter (MLP), located at 16.9 map units. The late mRNAs in Ad35 can be divided into five families of mRNAs (L1–L5), depending on which poly(A) signal is used by these mRNAs. Based on the MLP consensus initiator element, and splice donor and splice acceptor site sequences, the length of tripartite leader (TPL) was predicted to be 204 nucleotides. The first leader of the TPL, which is adjacent to MLP, is 45 nucleotides in length. The second leader located within the coding region of DNA polymerase is 72 nucleotides in length. The third leader lies within the coding region of precursor terminal protein (pTP) of E2B region and is 87 nucleotides in length. While Ad5 contains two virus-associated (VA) RNA genes, only one virus-associated RNA gene occurs in the genome of Ad35. This VA RNA gene is located between the genes coding for the 52/55K L1 protein and pTP.

Construction of Ad35 vectors and in vitro vector characterization

To study the potential of Ad35-based gene transfer, replication-defective vectors lacking either E1A or the entire E1 region were generated. Two E1A-deficient vectors, Ad35nbg and Ad35GFP, were generated. Ad35nbg contains the E1A promoter and E1A poly(A) signal controlling expression of the β-gal coding region. Ad35GFP contains the cytomegalovirus (CMV) immediate early promoter and simian virus 40 (SV40) poly(A) signal controlling expression of GFP. Both vectors grew to high titers in PER.C6 cells (Fallaux et al., 1998), with viral yields of more than 4000 particles per cell. A vector lacking both E1A and E1B regions, Ad35delE1, was generated and propagated in an E1, E2A, and E4 complementing cell line, A70.S54 (Gorziglia et al., 1999). Yields of Ad35delE1 were more than 2500 particles per cell.

To compare the gene transfer efficiency of Ad35-based

Table 1
In vitro Ad5- and Ad35-mediated transduction of human and mouse cell line[a]

Cell line	Ad5GFP	Ad35GFP
293	100 ± 1.2	100 ± 1.9
A549	60 ± 7.44	74 ± 3.2
PER.C6	100 ± 1.7	100 ± 1.1
H460	25 ± 4.2	41 ± 2.8
Hep3B	74 ± 4.8	66 ± 7.5
LNCaP	42 ± 5.2	61 ± 1.9
PC3	6 ± 0.2	48 ± 1.5
PC3M	71 ± 3.5	50 ± 2.7
CMT-64 (mouse)	47 ± 1.3	0.085 ± 0.007
JC (mouse)	23.4 ± 0.95	0.9 ± 0.03

[a] Cells were transduced at 25 particles per cell. Transduced cells were monitored for GFP expression by flow cytometry at 48 h postinfection. Data are presented as the mean percentage of cells expressing GFP from three experiments ± SD.

vectors with that of Ad5 vectors, a panel of established human and mouse cell lines were transduced with Ad35GFP or Av1GFP. Av1GFP is an E1/E3-deficient early-generation Ad5-based vector containing a GFP expression cassette under the control of Rous sarcoma virus (RSV) promoter, tripartite leader, and SV40 poly(A) signal. The transgene delivery into these cells was monitored by FACS analysis 48 h postinfection. At the tested multiplicity of infection, both Ad35GFP and Av1GFP were able to transduce 100% of 293 cells and PER.C6 cells (Table 1). Higher transduction efficiencies with Ad35GFP were observed in both in LNCaP and PC3 prostate tumor cell lines and in H460 human non-small cell lung cancer cells compared with the Ad5-based vector. Both the Ad35 and Ad5 vectors displayed similar gene delivery efficiencies in A549 human lung carcinoma cells and Hep3B liver carcinoma cells. Only one cell line, PC3M human prostate adenocarcinoma cells, displayed a higher transduction efficiency with the Ad5-based vector. Interestingly, no transduction of mouse JC, adenocarcinoma, and CMT-64 mouse lung carcinoma cells was observed with Ad35GFP, even at a dose of 1000 particles per cell. These data demonstrate the potential utility of Ad35-based vectors for human gene transfer.

In vivo Ad35 biodistribution, toxicity, and blood clearance rate

The biodistribution of Ad35 in mice was evaluated following intravenous administration of the wild-type virus at a dose of 1.25×10^{12} particles/kg, and compared with that of wild-type Ad5. The viruses were delivered to groups of 12 female C57B1/6 mice on Day 0. Groups of animals were sacrificed at Days 1 and 4 and selected tissues were analyzed for the presence of viral DNA by Southern analysis (data not shown) and a sensitive hexon-based real-time PCR assay (Table 2). Both quantitation methods displayed similar results. While Ad5 displayed 978 copies of viral DNA per cellular genome in the liver at Day 1, which declined to 44 viral DNA copies at Day 4, Ad35 displayed 6.5 copies of viral DNA per cellular genome in the liver at Day 1 which declined to less than 0.5 copy at Day 4. It is probable that the higher DNA content in the liver for both Ad5 and Ad35 at Day 1 may be due to uptake of the virus by Kupffer cells (Lieber et al., 1997; Worgall et al., 1997, Tao et al., 2001).

The relative hepatotoxicities of Ad5 and Ad35 were assessed by analysis of plasma from virus-treated animals for the presence of liver enzymes, aspartate aminotransferase (AST) (Fig. 3), alanine aminotransferase (ALT), and alkaline phosphatase (AP, data not shown). One day after treatment, mice that received Ad35 and Ad5 displayed 2- and 5-fold increases in AST levels, respectively. These levels returned to baseline by Day 4 for mice that received Ad35. In contrast, in mice treated with Ad5 these levels increased by 40-fold by Day 4. These data suggest that Ad35 may be less hepatotoxic than Ad5 in mice.

The blood clearance kinetics of Ad35 and Ad5 vectors were studied in mice following systemic delivery of vectors expressing GFP. Mice were bled at different time intervals, and aliquots of plasma were used to transduce cells. The amount of virus remaining in the blood was assessed by quantitation of GFP expression in transduced cells. The circulating levels of virus particles immediately after vector administration were estimated using the total number of injected transducing units and on the assumption that 7.3% of mouse body weight is blood (Alemany et al., 2000). Within the first 2 min of vector administration, large, 196-

Table 2
Biodistribution of Ad5 and Ad35 vectors in mice as determined by hexon based real-time PCR[a]

Day postinjection	Liver	Spleen	Lung	Heart	Kidney	Marrow
Ad5						
Day 1	978 ± 134.35	0.72 ± 0.33	0.69 ± 0.22	0.11 ± 0.09	0.06 ± 0.04	0.19 ± 0.13
Day 4	44.12 ± 47.28	0.57 ± 0.49	0.29 ± 0.08	0.06 ± 0.02	0.06 ± 0.04	0.29 ± 0.07
Ad35						
Day 1	6.45 ± 1.92	2.06 ± 0.36	0.29 ± 0.09	0.05 ± 0.02	0.05 ± 0.02	0.08 ± 0.04
Day 4	0.45 ± 0.26	0.07 ± 0.04	0.08 ± 0.06	0.02 ± 0.01	0.03 ± 0.03	0.02 ± 0.01

[a] The real-time PCR was performed as described under Materials and Methods. Quantification of adenovirus copy number was performed using a standard curve consisting of dilutions of adenovirus DNA from 1,500,000 copies to 15 copies in 10 ng of mouse genomic DNA. The average number of total copies was normalized to copies per cell based on the amount of input DNA calculated using a genome size of 6×10^9 bp. Data are displayed as means ± SD.

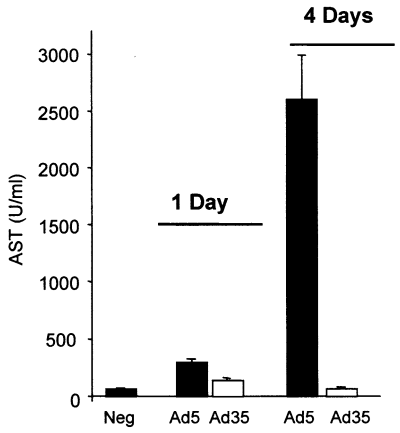

Fig. 3. Ad35 hepatotoxicity in mice. Aspartate aminotransferase (AST) levels were measured in the plasma of mice ($n = 6$) treated with 1.25×10^{12} particles/kg wt Ad5 or wt Ad35 virus. Plasma was collected on Days 1 and 4. Filled bars represent Ad5 and open bars represent Ad35. The bar labeled "Neg" represents pretreatment AST levels ($n = 3$). The mean values and standard deviations are displayed.

and 306-fold reductions in Ad35 and Ad5 vector titers, respectively, were observed (Fig. 4). After this initial rapid decline, the circulating levels of Ad5 and Ad35 reduced by half in 4.97 and 11.95 min respectively. Therefore, 2 min following vector administration, the circulation time of Ad35 in mice was approximately 2.4 times longer than that of Ad5. The clearance rates of Ad5 and Ad35 in mouse blood were similar 30 min after vector administration. As a control, to rule out direct inactivation of vectors by blood components, separate aliquots of the vectors were incubated with heparinized mouse blood for 1 h prior to separation of plasma after which A549 cells were transduced. No significant drop in vector titers was observed (data not shown). Taken together, the in vitro mouse cell line transduction results, the in vivo biodistribution, and blood clearance analyses suggest that mice do not contain the Ad35 cell surface receptor.

Ad35 cell entry mechanism

To understand the entry pathways used by Ad35 and to determine if Ad35 uses CAR as a primary receptor, competition assays with Ad5 fiber protein were performed (Fig. 5). Ad35 entered A549 cells equally efficiently with or without blocking of CAR. In contrast, Ad5 entry was greatly inhibited when fiber was used to compete for CAR binding. These data demonstrate that Ad35 transduction is CAR-independent.

Discussion

In this work, we evaluated Ad35 as a novel serotype for gene transfer vector development. Ad35 was chosen for several reasons, including favorable growth characteristics and broad in vitro tropism. Foremost, Ad35 displayed limited penetrance in the human population. Indeed, screening

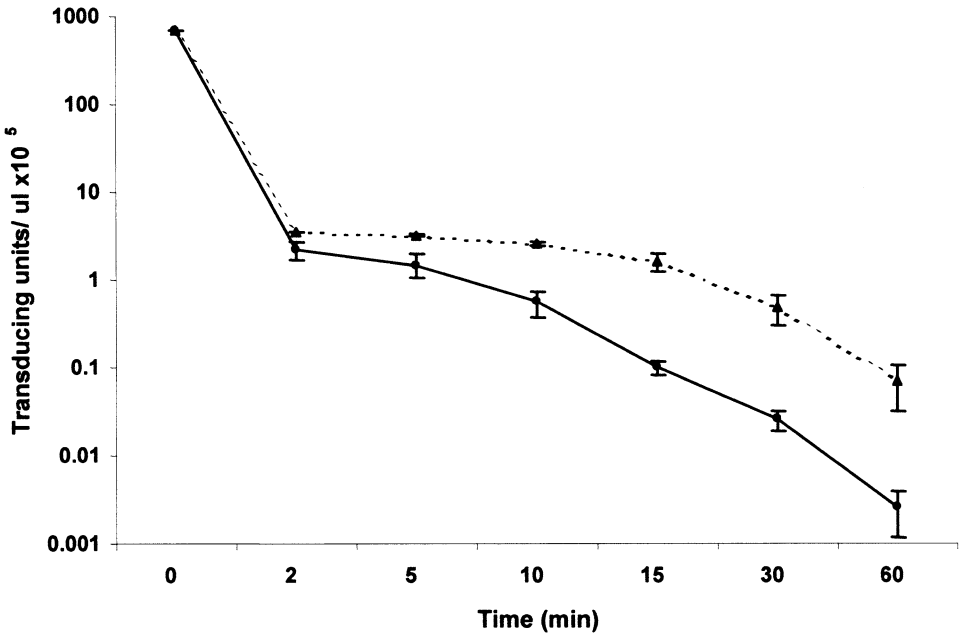

Fig. 4. Circulation time of Ad5 and Ad35 in mouse blood. Mice in cohorts of three were treated with 3×10^{12} particles/kg of either Av1GFP or Ad35GFP and bled at the indicated times. Plasma was used to transduce A549 cells, cells were harvested, and GFP expression was quantitiated by flow cytometry to determine the transducing particles per microliter of plasma. Vector input in transducing units at T0 was calculated by dividing the total injected particles with the total amount of blood in mice assuming 7.3% of body weight of mouse is blood (Alemany et al., 2000). ●, Ad5; ▲, Ad35. Data are plotted as mean values and standard deviations.

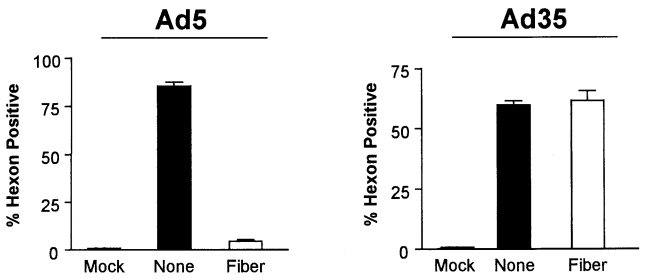

Fig. 5. Cell transduction competition analyses. A549 cells were transduced with 200 particles per cell of Ad5 or Ad35, in the absence (None) or presence of Ad5 fiber protein (Fiber) or mock-transduced (Mock). Transduction was quantitated as the percentage of cells that were hexon positive following incubation with a FITC-conjugated hexon antibody and analysis by flow cytometry. Data are plotted as mean values and standard deviations.

of 200 human serum samples collected from around the world revealed that the prevalence of neutralizing antibodies to Ad35 was only 17% compared with more than 50% for Ad5. These data are consistent with a previous study (Flomenberg et al., 1987). Interestingly, the majority of Ad5-positive serum samples had high neutralizing antibody titers, whereas the vast majority of Ad35-positive serum samples had low titers. These observations suggest that Ad5 infections in humans not only are widespread but may also be recurrent or persist as latent infections. However, it is currently unclear why the majority of humans lack neutralizing antibodies to Ad35, especially since in vitro analyses revealed that Ad35 transduced the vast majority of the human cell lines as well as or better than Ad5. Ad35 was isolated from immunocompromised patients (Flomenberg et al., 1987), suggesting that the virus may not replicate effectively in the presence of a normal immune system.

The E3 region of subgroup C adenoviruses is thought to function in modulation of the host immune system (Wold et al., 1999). However, the E3 region of Ad35 differs from that of Ad5 in several ways. For example, the promoter of the Ad35 E3 region lacks a second NF-κB binding site, suggesting a difference in the mechanism by which the E3 promoter is activated following immune stimulation and in the lymphoid tissue (Basler and Horwitz, 1996). In addition, the E3 region of Ad35 has the potential to encode two proteins, 20.3 and 20.6 kDa, the purposes of which are currently unknown (Basler and Horwitz, 1996). Finally, the Ad35 E3 region lacks the adenovirus death protein (Basler and Horwitz, 1996), which plays a major role in the release of Ad2 from infected cells (Tollefson et al., 1996). Taken together, the differences in the E3 regions of Ad5 and Ad35 may influence the immune response following exposure to these serotypes in the human population.

The availability of the complete nucleotide sequence of Ad35 from GenBank was useful and allowed us to construct a tentative genome organization map based on sequence homology to other adenovirus serotypes. Such a map was critical for the generation of several E1- and E1A-deficient vectors. The genome organization of Ad35 is similar to that of Ad5 with minor differences. Unlike Ad5, which has two VA RNA genes, Ad35, similar to other serotypes in subgroups A, F, and B2 (Ma and Mathews, 1996), has only one VA RNA gene, RNAI. Adenoviral VA RNAI was shown to stimulate protein synthesis in infected cells (O'Malley et al., 1989) and antagonize the interferon-induced host antiviral defense system. Another striking difference between Ad5 and Ad35 is that the Ad35 fiber protein, like other subgroup B viruses, is short, 323 amino acids (aa), and contains only six repeats in the shaft region (Basler et al., 1996). In contrast, the Ad5 fiber is 581 aa and contains 22 repeats in the shaft (Chroboczek et al., 1995). Interestingly, the Ad35 fiber knob, similar to other subgroup B viruses (Roelvink et al., 1998; Shayakhmetov et al., 2000), does not contain the coxsackie B virus and adenovirus receptor (CAR) binding motif. Many viruses, such as those belonging to subgroups A, C, and E, do contain this motif (Bergelson et al., 1998; Roelvink et al., 1998). These observations suggest that Ad35 does not use the CAR cell surface receptor for cell entry. Indeed, we found that purified Ad5 fiber protein did not inhibit Ad35 cell transduction. The Ad35 penton base, like that of Ad5, contains the arginine–glycine–aspartic acid (RGD) motif that has been implicated in integrin binding (Wicham et al., 1993; Li et al., 2002), suggesting that, similar to Ad5, Ad35 may use integrins for internalization. Identification of the specific Ad35 cell surface receptor is currently under investigation.

In vitro transduction studies revealed that Ad35 transduced the majority of human cell lines tested as well as or better than Ad5, suggesting that the specific receptor for Ad35 is abundantly expressed on human cells and may be ubiquitous. In contrast to the in vitro human cell line transduction data, transduction of two mouse cell lines was extremely inefficient. Likewise, investigation of the biodistribution of Ad35 following systemic administration in mice revealed that all mouse tissues analyzed, including liver, lung, heart, kidney, and bone marrow, showed extremely low transduction. Not surprisingly, Ad35 displayed low hepatotoxicity, most likely due to the low transduction efficiency of mouse liver. Finally, the circulation time of Ad35 in mouse blood was two- to threefold longer than that of Ad5. Taken together, these data demonstrate that Ad35 does not efficiently transduce mouse cells in vitro or in vivo and suggest that rodents may lack the Ad35 cell surface receptor.

In the present study, it was demonstrated that Ad35 vectors lacking only the E1A region could be generated and propagated in PER.C6 cells, cells that constitutively express the E1 proteins of Ad5. These data suggest that E1A proteins of Ad5 can complement those of Ad35. Similarly, it was demonstrated previously that the E1A proteins of Ad5 could complement human adenovirus serotypes belonging to both subgroups C and B (Abrahamsen et al., 1997), as well as bovine and porcine adenoviruses (Reddy et al., 1999a,b). In contrast, the Ad35 vector lacking both the E1A

and E1B coding regions could not be generated and grown in PER.C6 cells, suggesting that the E1B proteins of Ad5 could not complement Ad35. This limitation was overcome by using a complementing cell line that expressed both the E4 proteins and the E1 proteins of Ad5. Such a cell line, A70.S54 was developed previously for use in the generation of Ad5-based vectors lacking the E1, E2A, and E4 regions (Gorziglia et al., 1999). Ornelles and Shenk (1991) reported that the Ad5 E1B 55-kDa protein forms a complex with the Ad5 E4 ORF6 protein, which, in turn, binds a component of the host nucleocytoplasmic transport system to promote selective transport of late viral mRNA. We hypothesized that the use of E1B and E4 proteins originating from the same serotype was required for E1B 55-kDa and E4 ORF6 association. Indeed the lack of complementation of the E1A- and E1B-deficient Ad35 vector in PER.C6 cells and the efficient propagation of this vector in A70.S54 cells suggest that the Ad5 E1B 55-kDa protein and the Ad35 E4 ORF6 protein are not capable of complex formation, resulting in impaired transport of late viral mRNA.

The data presented in this report, including limited immunity to Ad35 in the human population, favorable growth characteristics, highly efficient transduction of human cells, and the development of replication-defective vectors, demonstrate the great potential of Ad35 for human gene therapy applications.

Materials and methods

Viruses and cells

The Holden strain of human adenovirus type 35 (Ad35) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was cultivated in PER.C6 cells (Fallaux et al., 1998). The cells were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) and 10 mM magnesium chloride (Sigma, St. Louis, MO, USA). Virus was purified by two rounds of CsCl gradients: a one-step gradient followed by one continuous gradient centrifugation. The purified virus concentration was determined spectrophotometrically, assuming $1A_{260} = 1.1 \times 10^{12}$ particles (Mittereder et al., 1996). Viral genome integrity was confirmed by restriction enzyme analysis of DNA isolated from CsCl-purified vectors. Titers of purified viruses were also determined by a standard plaque assay using PER.C6 cells. The yields of wild-type (wt) Ad35 from PER.C6 cells were greater than 20,000 particles per cell, with particle to PFU ratios less than 15. An E1, E2A, and E4 complementing cell line, A70.S54 (Gorziglia et al., 1999), was used for generation and cultivation of the E1 deletion mutant of Ad35. The cells were grown in improved minimum essential medium (IMEM, Biosource International, Rockville, MD, USA) supplemented with 5% fetal bovine serum and 1 μg/ml tetracycline. For virus production, these cells were induced by addition of dexamethasone and removal of tetracycline from the medium.

Human serum samples and neutralizing antibody assays

Two hundred randomly collected human sera samples were used in neutralization assays. These samples were purchased from Bioheme (Salt Lake, UT, USA). Of these, 100 came from donors in the United States, 50 from Europe, and 50 from Japan. To inactivate complement, all serum samples were heated to 56°C for 30 min. To perform the neutralizing antibody assay, the day before the experiment, PER.C6 cells were seeded at a density of 3×10^4 cells per well in a 96-well tissue culture plate. On the day of the experiment, each serum sample was serially diluted (from 1:2 to 1:256) in infection medium (2% FBS in DMEM, 10 mM $MgCl_2$). The serial dilution of each serum sample (50 μl) was added to an equal volume of virus (100 $TCID_{50}$). As a control, FBS was also serially diluted and combined with 100 $TCID_{50}$ of adenovirus. The virus/serum dilutions were incubated for 1 h at 37°C. Medium was removed from the PER.C6 cells and replaced with the virus/serum mixtures and incubated for 1 h at 37°C. Control wells were infected with equal amounts of virus (100 $TCID_{50}$) without addition of serum. The infection mixture was then removed and replaced with growth medium and incubated for 10 days until CPE occurred in the control wells. The neutralizing antibody titer was scored as the reciprocal of the highest serum dilution that could prevent CPE formation in PER.C6 cells.

Construction of E1a-deficient Ad35 vectors

First, an Ad35 full-length clone was constructed. The left (985 bp) and right (5403 bp) terminal fragments generated by digestion of Ad35 genomic DNA with *Pst*I were cloned separately into pGEM-3S. Plasmid pGEM-3S was derived from pGEM-3Z (Promega, Madison, WI, USA), by introduction of a I-*Sce*I recognition sequence into the polylinker. The nucleotide numbers of the Ad35 genome referred to in this report are according to GenBank Accession No. AX049983. The cloned terminal fragments with upstream I-*Sce*I sites were then inserted into pBlueScript (Stratagene, La Jolla, CA, USA) to generate pBSAd35L&RTF. The plasmid pFLAd35, which contains the full-length Ad35 genome bordered by I-*Sce*I sites, was generated by combining the *Pst*I-linearized pBSAd35L&RTF and the genomic DNA of wild-type Ad35.

The vector Ad35GFP, which contains a GFP expression cassette replacing the E1a region of Ad35, was generated in several steps. First the plasmid pFLAD35, containing the entire Ad35 genome in pBlueScript, was digested with *Sph*I, and the fragment containing the left and right terminal fragments (nucleotides (nt) 1–3094 and 32,683–34,794, respectively) of Ad35 was gel purified and self-ligated to generate

pAd35L&RSph. By using polymerase chain reaction (PCR), 1115 bp corresponding to the promoter and coding sequences of E1A between bp 338 and 1454 were deleted and a recognition sequence for *Pme*I was inserted into pAd35L&RSph to generate pAd35L&RsphdelE1A. The gene coding for GFP under the control of CMV immediate early promoter and SV40 poly(A) signal obtained from pEGFP (Clontech, Palo Alto, CA, USA) was cloned into the *Pme*I site of pAd35L&RSphdelE1A to generate pAd35L&RsphdelE1AGFP. The plasmids pFLAd35delE1A and pFLAd35delE1AEGFP, which contain the full-length Ad35 DNA with the E1A deletion and GFP coding region replacing the E1A coding region, respectively, were generated by combining *Sph*I-linearized pAd35L&RsphdelE1A and pAD35L&RSphGFP and the genomic DNA of wild-type Ad35, respectively. To generate the viruses Ad35v0.5 and Ad35GFP, pFLAD35delE1A and pFLAd35delE1AGFP respectively were digested with I-*Sce*I and transfected into PER.C6 cells. The E1 deletion mutant of Ad35 was also generated in several steps. First the plasmid pFLAd35delE1A was digested with *Nru*I, and the fragment containing the left and the right terminal fragments of Ad35 was gel purified and self-ligated to generate pAd35L&RNrudelE1A. By using *Pme*I and *Pac*I restriction enzyme digestions, a 2579-bp fragment (between bp 338 and 2917) spanning the E1A poly(A) signal, complete open reading frame (ORF) of E1B 19 kDa, and first 1001 bp of the ORF of E1B 55 kDa was deleted from pAd35L&RNrudelE1 to generate pAd35L&RNrudelE1. The plasmid pFLAd35delE1, which contains the full-length Ad35 DNA with the E1 deletion, was generated by combining *Nru*I-linearized pAd35L&RNrudelE1 and the genomic DNA of wild-type Ad35. To generate the virus Ad35delE1, pFLAd35delE1 was digested with I-*Sce*I and transfected into A70.S54 cells (Gorziglia et al., 1999). A70.S54 is a stable cell line derived from AE1-2a cells (Gorziglia et al., 1996) and expresses E1, E2a, and E4 proteins of Ad5. The transfection of I-*Sce*I-linearized pFLAd35delE1 into PER.C6 cells did not result in virus generation.

The vector Ad35nbg, which contains a β-galactosidase expression cassette under the control of E1A promoter and poly(A) signal, was generated in several steps. First, the plasmid pAd35L&RSphnbg was generated following deletion of E1A coding region (892 bp; from nt 559 to 1451) from pAd35L&RSph and insertion of a DNA fragment encoding β-galactosidase. Next, the plasmid, pFLAd35delE1Anbg was generated by combining the wild-type Ad35 genomic DNA and *Sph*I-linearized pAd35L&RSphnbg. The vector Ad35nbg was generated in PER.C6 cells following transfection of I-*Sce*I-linearized pFLAd35delE1Anbg.

Animals and study design

Female C57B1/6 mice, 4–6 weeks old, purchased from Harlan Sprague Dawley (Indianapolis, IN, USA), were used in the study. For the biodistribution study, doses of 1.25×10^{12} particles/kg wt Ad5 and wt Ad35 viruses were delivered intravenously using the lateral tail vein in a dose volume of 10 ml/kg on Study Day 0. Blood was collected into sodium citrate solution by orbital sinus bleeds on Days −1, 1, and 4 of virus administration. At the time of necropsy, liver, spleen, kidney, lungs, heart, and bone marrow (femur) were collected and used for genomic DNA extraction. The plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AP) were measured by AniLytics, Inc. (Gaithersburg, MD, USA). To compare the blood circulation time of Ad5 to Ad35 in mice, mice ($n = 3$) were treated with 3×10^{12} particles/kg of either Av1GFP or Ad35GFP and bled at 2, 5, 10, 15, 20, 30, and 60 min postinjection. The animals were sacrificed following the 60-min bleed. Plasma was separated from the blood immediately after collection, diluted in infection medium, and used to transduce A549 cells. The cells were harvested and fixed 24 h postinfection and FACS analysis was performed to detect the fraction of GFP-positive cells. All animal procedures were performed at Genetic Therapy, Inc., in an AAALAC-accredited animal facility in accordance with the Animal Welfare Act and institutional guidelines for the humane treatment of laboratory animals.

Organ DNA analyses

Genomic DNA was isolated from collected organs using the Qiagen Blood and Cell Culture DNA midi kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. To determine the Ad5 DNA content in each tissue, a real-time PCR assay was used as described by Smith et al. (2002). Ad35 viral DNA content in DNA collected from mouse organs was determined by Ad35 Hexon Taqman real-time PCR assay. PCR primers and a Taqman probe specific to Ad35 hexon sequences were designed using Primer Express software Version 1.0 (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences were: (hexon forward) primer 5′-TACATGCACATCGCCGGA-3′, (hexon reverse primer) 3′-CGGGCGAACTGCACCA-3′, and (hexon probe) 5′-FAM-ATGCTTCGGAGTACCTGAGTCCGGG-TAMRA-3′. Amplification was performed in a reaction volume of 50 μl under the following conditions: 10 ng of sample DNA, 1X Taqman Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 300 nM reverse primer, and 100 nM hexon probe. Thermal cycling conditions were: 2-min incubation at 50°C, 10 min at 95°C, followed by 40 cycles of successive incubation at 95°C for 15 s and 60°C for 1 min. Data were collected and analyzed using the 7700 Sequence Detection System software version 1.6.3 (Applied Biosystems). Quantification of adenovirus copy number was performed using a standard curve consisting of dilutions of adenovirus DNA from 1,500,000 copies to 15 copies in 10 ng of mouse DNA. The average number of total copies was normalized to copies per cell based on the input DNA weight amount and a genome size of 6×10^9 bp.

In vitro cell transduction and competition analyses

To determine and compare the transduction efficiency of Ad35GFP with that Av1GFP, selected human cell lines were transduced with these two vectors at 25 particles per cell (PPC) for 2 h at 37°C in a total volume of 0.5 ml of culture medium containing 2% heat-inactivated fetal bovine serum (FBS) in six-well plates. The infection medium was replaced with 2 ml of the appropriate culture medium containing 4% FBS. The cells were incubated for 48 h to allow for GFP expression. The cell monolayer was then detached by trypsin treatment, washed, and fixed. The percentage of transduction was determined by flow cytometry. Each vector transduction was carried out in triplicate and the average percentage of transduction determined. Human embryonic kidney cells, 293, were cultured in DMEM containing 10% FBS. Human non-small cell lung carcinoma (H460) and human prostate cancer (PC3M-2AC6) cells were cultured in RPMI-1640 medium containing 10% FBS. Human hepatocellular carcinoma (Hep3B) cells, were cultivated in minimal essential medium containing 10% FBS. Human prostate cancer (LNCaP) cells were cultured in RPMI containing 10% FBS, L-glutamine (2 mM), nonessential amino acids (0.1 mM), sodium carbonate (0.075%), and sodium pyruvate (1 mM). Human prostate (PC3) cancer cells were cultured in RPMI supplemented with 10% FBS and L-glutamine (2 mM). CMT-64 is a murine small cell lung carcinoma and was maintained in DMEM containing 10% FBS. Murine mammary adenocarcinoma (JC) cells were cultured in RPMI containing 10% FBS.

For the competition analyses, the day before the experiment, A549 cells were seeded at a density of 4×10^5 cells per well in a 12-well tissue culture dish in the growth medium (Richter's medium containing 10% FBS). To determine the role of CAR in virus entry, duplicate wells of cells were incubated with infection medium (Richter's medium plus 2% FBS) containing purified Ad5 soluble fiber protein (20 ug/well) at room temperature for 15 min. Virus particles corresponding to 200 ppc for Ad5 and Ad35 were then added to the cells and the plates were further incubated at 37°C for 1 h, after which 1 ml of fresh growth medium was added, and plates were further incubated at 37°C for 24 h. The following day, cells were fixed with paraformaldehyde, permeabilized by treating with Tween 20, stained with anti-hexon mouse monoclonal antibodies conjugated to FITC (MAB8052, Chemicon International Temecula, CA, USA), and analyzed by flow cytometry.

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