Porcine Adenoviral Vectors Evade Preexisting Humoral Immunity to Adenoviruses and Efficiently Infect Both Human and Murine Cells in Culture

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ABSTRACT

Preexisting immunity against human adenoviruses (HAd) limits the efficiency of transduction of HAd vectors in humans. In addition, development of a vector-specific immune response following the first inoculation with a HAd vector further lowers vector uptake following readministration. We investigated the usefulness of porcine adenovirus serotype 3 (PAd3)-based vectors as a supplement to HAd vectors. Here we demonstrate that preexisting HAd-specific neutralizing antibodies in humans do not cross-neutralize PAd3. In order to generate E1A-deleted PAd3 vectors, an E1-complementing cell line of porcine origin was produced. PAd-GFP (E1A-deleted PAd3 vector expressing green fluorescent protein; GFP) and HAd-GFP (E1-deleted HAd5 vector expressing GFP) transduced human cell lines with comparable efficiencies. Both of these vectors efficiently transduced murine MT1A2 breast cancer cell line, while PAd-GFP transduced murine NIH-3T3 fibroblast cell line significantly better (*P*<0.05) than HAd-GFP. These results suggest that PAd3 vectors would be promising supplement to HAd vectors as a delivery vehicle for recombinant vaccines and gene therapy applications.

Key Words: Porcine adenoviral vectors, nonhuman adenoviral vectors, adenoviral vectors, gene therapy, delivery vehicle, circumvention of vector immunity, preexisting immunity

1. INTRODUCTION

Human adenoviral (HAd) vectors have demonstrated tremendous potential as a gene delivery system for applications ranging from recombinant vaccines to cancer therapy (Alemany et al., 2000; St George, 2003). One of the main impediments to clinical use of HAd vectors is the vector immunity. Due to the ubiquitous nature of HAd, a large percentage of the general population is exposed to one or more HAd serotypes at multiple times in their lives (Horwitz, 2001). This leads to the development of HAd-specific immune response of variable degree in the majority of human population. This preexisting vector immunity interferes with the expected success even on the first inoculation with the vector. Moreover, all adenoviral vectors (replication-competent, replication-defective, conditional replication-competent, and helper-dependent or gutless) result in vector-specific immune responses that could significantly reduce vector uptake on readministration (Akbulut et al., 2003; Hackett et al., 2000).

Following decline in transgene expression to background levels, readministration of the vector is necessary to maintain the therapeutic effect (Vlachaki et al., 2002). Therefore, it is important to develop strategies that could effectively elude vector immunity. Some of these strategies include immunosuppression (Guibinga et al., 1998; Kay et al., 1995; Ye et al., 2000), modification of immunodominant capsid epitopes (Morral et al., 2002), use of different HAd serotypes (Mack et al., 1997; Parks et al., 1999; Reddy et al., 2003) retargeting of HAd vectors by masking the adenoviral fiber knob to ablate binding to the coxsackievirus adenovirus receptor (CAR) (Douglas et al., 1999), targeting specific cell receptor by genetic modification of HAd fiber (Belousova et al., 2002; Bernt et al., 2003) and vector encapsulation into microparticles (Sailaja et al., 2002). In addition, use of nonhuman adenoviruses as vectors for eluding HAd immunity has been proposed (Hofmann et al., 1999; Kremer et al., 2000; Mittal et al., 1995; Reddy et al., 1999a) Several nonhuman adenoviruses are naturally nonpathogenic to human, and have been well characterized. Unlike human adenoviruses, it is expected that immunity to nonhuman adenoviruses will not be prevalent in the human population. It has been shown that preexisting humoral immunity in human did not cross-neutralize ovine adenovirus (Hofmann et al., 1999), chimpanzee adenovirus (Farina et al., 2001), or canine adenovirus (Kremer et al., 2000). Although we have previously demonstrated that HAd5- or porcine adenovirus serotype 3 (PAd3)-specific neutralizing antibodies raised in rabbits or mice were not cross-neutralizing (Moffatt et al., 2000), it is not known whether the preexisting adenoviral neutralizing antibody response in human will cross-neutralize PAd3.

Here we describe that preexisting HAd-specific neutralizing antibodies in human do not cross-neutralize PAd3. In addition to the development of adenoviral E1-expressing cell line of porcine origin and generation of replication-defective PAd3 vectors, this study compares transduction efficiencies of PAd3 and HAd5 vectors in human and murine cells in culture. Taken together, the results described in this manuscript are an important step towards harnessing the potential of PAd3 vectors as a delivery system for recombinant vaccines and gene therapy.

2. MATERIALS AND METHODS

2.1. Cell lines and viruses

293, 293*Cre* (constitutively expresses *Cre* recombinase enzyme; a gift from Merck, Inc., Whitehouse Station, NJ) (Chen et al., 1996), BHH-2C (bovine-human hybrid) (van Olphen and Mittal, 2002), MDA-MB-231 (aggressive human breast cancer), NIH-3T3 (murine fibroblast), MTIA2 (murine breast cancer; kindly provided by Dr. William Muller, Department of Biology, McMaster University, Hamilton, Ontario, Canada.) (Addison et al., 1995), PK-15 (pig kidney) and ST (swine testicle) cell lines were grown as monolayer cultures in Eagle's minimum essential medium (MEM) (Life Technologies, Gaithersburg, MD) supplemented with 5-10% reconstituted fetal bovine serum (FetalClone III; Hyclone, Logan, UT) and 50 µg/ml gentamicin. Human normal mammary epithelial cells (MCF-10A) were propagated as described (Noblitt et al., 2004). Fetal porcine retinal cells (FPRT) and FPRT-transformed with adenoviral E1 (FPRT-HE1-5) were grown in MEM supplemented with FetalClone III and gentamicin. The virus purification by cesium chloride-density gradient centrifugation was done as previously described (Graham and Prevec1991). The wild type (wt) or recombinant HAd5 or PAd3 were titrated in 293 or FPRT-HE1-5 cells, respectively by plaque assay.

2.2. Virus neutralization Assays

50 randomly collected normal human serum samples were kindly provided by Dan Follas, Follas Laboratories, Inc., Indianapolis, IN and were used as per the guidelines of institutional biosafety committee. All samples were incubated at 56°C for 30 min to inactivate complement. Serial, two-fold dilutions of each serum sample in 96-well plates were reacted with 100 p.f.u. of HAd5 or PAd3 for 1 h at 37°C followed by addition of 10^4 appropriate cells in each well. HAd5 or PAd3 neutralizing assay was done in BHH-2C or FPRT HE1-1-5 cells, respectively. The plates were incubated at 37°C for 5-7 days until complete cytopathic effect (c.p.e.) appeared in virus controls. Anti-HAd5 and anti-PAd3 hyperimmune sera raised in rabbits (Moffatt et al., 2000) against purified HAd5 and PAd3 preparations, respectively were used as positive serum controls. The virus neutralizing antibody titer was the reciprocal of the highest serum dilution that completely prevented the development of c.p.e.

2.3. Stable transformation of FPRT cells with HAd5 E1

FPRT cells grown in 60 mm dishes were transfected with pPGKHE1, a plasmid that carries HAd5 E1 region under the control of the phosphoglycerate kinase (PGK) promoter or pCMVHE1, a plasmid that contains HAd5 E1 region with the cytomegalovirus (CMV) immediate early promoter (van Olphen et al., 2002) using Lipofectin (Invitrogen Corporation, Carlsbad, CA)-mediated transfection as per the supplier's recommendations.

2.4. Construction of plasmids containing PAd3 sequences

All the procedures for constructing recombinant plasmids were as per the standard molecular biology protocols with minor modifications. The description of nucleotides and restriction sites is based on the genome sequence and transcription map of PAd3 (Genbank accession # AF083132; (Reddy et al., 1998).

2.4.1. Shuttle plasmids for generating PAd3 vectors

Approximately 430 bp right end fragment (nt 1-433) and 1494 bp left end fragment (nt 32600-34094) of PAd3 genome were amplified from wt PAd3 genomic DNA by PCR using specific primer sets containing *Pac*I recognition sequence. PCR products were cloned into pPolyII (Kindly provided by Transgene, Strasbourg, France) at *Pac*I site by three-way ligation to obtain pDS1.

To delete E1A region, a site directed-mutagenesis approach was adopted. PAd3 genome between nt 401 and 529 was amplified by PCR using specific primer set and plasmid pPAd3-E1(Aggarwal and Mittal, 2000) DNA as template. Similarly the genomic region between nt 1224 and 2023 was also amplified. The first PCR product was digested with *Eag*I and *Avr*II and the second was cleaved with *Avr*II and *Stu*I. These products were inserted into pDS1 at the *Eag*I-*Stu*I site by three-way ligation to generate pDS2. This plasmid carried the left and right end genomic sequences of PAd3 with a 695 bp deletion in E1A region and a unique *Avr*II site to facilitate cloning of any foreign gene in the E1A deletion region. The GFP gene of jellyfish (*Aequoria victoria*), flanked by the CMV promoter and the BGH polyadenylation signal obtained from pDC311-GFP (described below) was cloned into pDS2 at the *Avr*II site to obtain pDS2-GFP.

2.4.2. PAd3 genomic plasmids

Using homologous recombination in *E. coli* BJ5183, as described earlier for bovine adenovirus (van Olphen and Mittal, 1999), three genomic plasmids, pPAd3 (full-length genomic plasmid), pPAd-ΔE1A (genomic plasmid with E1A deletion), or pPAd-GFP (genomic plasmid with GFP cassette in E1A region) were generated, by cotransformation of *E. coli* with wt PAd3 genomic DNA and *Stu*I-linearized pDS1, pDS2 ,or pDS2-GFP, respectively.

2.4.3. Rescue of E3 deletion in genomic plasmids

To rescue E3 deletion in the genomic plasmids, the 2.5 kb *Kpn*I-*Spe*I fragment obtained from pMNeP3.10 [a plasmid containing *Bam*HI 'B' fragment of PAd3 (nt 18664-31064) with a 622 bp deletion in the E3 region (Aggarwal and Mittal, unpublished)] was used for co-transformation of *E. coli* BJ5183 along with *Sna*BI-digested pPAd-ΔE1A or pPAd-GFP to obtain pPAd-ΔE1AE3 or pPAd-GFP2, respectively.

2.5. Plasmids for generating HAd vectors

The pDC316 (a plasmid containing the packaging signal and the left end genomic sequences of HAd5 with a murine CMV promoter and SV40 polyadenylation sequences followed by a *lox*P site for specific homologous recombination), pBHGloxΔE1,3Cre (a plasmid containing almost entire HAd5 genome and a *lox*P site except the packaging signal, E1 and E3 deletions) and pDC311 (a plasmid containing left end sequence and packaging sequence of HAd5 and a *lox*P site) were obtained from Mircobix Biosystem, Inc. Ontario, Canada. A 1.1 kb fragment containing CMV promoter and bovine growth hormone (BGH) polyadenylation sequences was excised from pcDNA3 (Invitrogen Corp., Carlsbad, CA) and cloned into pDC311 at the *Stu*I site to generate pDC311-CMV. The GFP gene was inserted at the *Not*I site of pDC311-CMV to obtain pDC311-GFP.

2.6. Generation of PAd and HAd vectors

FPRT HE1-5 cells in monolayer cultures in 60-mm dishes were transfected with 5 µg of *Pac*I-digested PAd genomic vectors: pPAd3, pPAd-ΔE1A, pPAd-GFP, pPAd-ΔE1AE3 and pPAd-GFP2 using Lipofectin-mediated transfection as per the supplier recommendations to generate rPAd3, PAd-ΔE1A, pPAd-GFP, PAd-ΔE1AE3 and PAd-GFP2, respectively. Recombinant virus-induced c.p.e. was visible in 2-3 weeks post-transfection. Each recombinant virus was plaque purified in FPRT HE1-5 cells before further characterization.

293*Cre* cells were cotransfected with pDC316 or pDC311-GFP and pBHGloxΔE1,3 Cre using calcium-phosphate technique (Graham and van der Eb, 1973) to generate HAd-ΔE1E3 and HAd-GFP, respectively by the *Cre* recombinase-mediated site-specific recombination (Ng et al., 1999). HAd-GFP and HAd-ΔE1E3 were plaque purified in 293 cells.

2.7. Immunoprecipitation and Western blot analyses
These analyses were performed essentially as described previously (van Olphen et al., 2002).

2.8. GFP flow cytometry and fluorescence microscopy
Mock or vector-infected cells were harvested by trypsinization and counted and resuspended in phosphate-buffered saline (PBS), pH 7.2 at a concentration of 10^6 cells per ml. GFP expressing cells were sorted using a Coulter XL-MCL cytometer at the Purdue Cytometry Laboratories. For fluorescence microscopy, cells grown on coverslip cultures were washed with PBS, fixed using 3.7 % formaldehyde and observed under Nikon Eclipse E400 fluorescence microscope using FITC filters. Pictures were captured using a Spot RT camera (Diagnostic Instruments, Sterling heights, MI).

2.9. Immunofluorescence
Cells grown in 8-well Lab-Tek chamber slides were fixed and permeabilized with 0.1 % Triton X-100 and incubated with primary (rabbit anti-CAR) antibody and secondary (FITC-conjugated goat anti-rabbit IgG) antibodies. The slides were visualized under a fluorescence microscope as described above.

3. RESULTS

3.1. Absence of PAd3 cross-neutralizing antibodies in human
It is well known that the majority of human population has varying levels of preexisting HAd immunity that could significantly inhibit HAd vector entry in target cells (Dong et al., 1996). We observed that HAd5-, BAd3- or PAd3-neutralizing antibodies were not cross-neutralizing (Moffatt et al., 2000). It is not known whether neutralizing antibodies against some of the >50 HAd subtypes will cross-react with PAd3 or whether exposure to PAd subtype/s occurs naturally in human. To determine whether adenovirus-neutralizing antibodies in human will cross-neutralize PAd3, 50 randomly collected human serum samples were tested for HAd5 or PAd3 cross-neutralizing antibodies by virus neutralization assays. Hyperimmune anti-HAd5 or anti-PAd3 rabbit sera were used as positive controls. HAd5-specific neutralizing titers above 4, 16, 64 and 256 were observed in approximately 94, 76, 42 and 16% of the samples, respectively (Fig. 1). None of the serum samples showed PAd3 cross-neutralizing titer of 4 (the lowest serum dilution in our assay) or above (Fig. 1) suggesting that preexisting neutralizing antibodies against PAd3 are not prevalent in human population and HAd neutralizing antibodies did not cross-neutralize PAd3. These findings strongly favor the development of PAd3 vectors for gene transfer in human.

3.2. Isolation of a porcine cell line expressing HAd5 E1
For generating E1A-deleted PAd3 vectors there was a requirement of a cell line of porcine origin that constitutively expresses E1 proteins. The HAd5 E1A gene is known to complement E1A functions of porcine and bovine adenoviruses (Reddy et al., 1999b; van Olphen et al., 2002; Zheng et al., 1994) PAd3 and BAd3 infect human cells in culture without active virus replication (Dong et al., 1996; Farina et al., 2001; Hofmann et al., 1999; Kremer et al., 2000; Mittal et al., 1995; Moffatt et al., 2000; Reddy et al., 1999a; Reddy et al., 1999b; Zheng et al., 1994) (Bangari and Mittal, unpublished). We and others have isolated cell lines expressing HAd5 E1 by transfecting human (Fallaux et al., 1996) or bovine (van

Olphen et al., 2002) embryonic retinoblasts with a plasmid containing HAd5 E1. To obtain an E1-expressing porcine cell line, we transfected FPRT cells with a plasmid containing HAd5 E1 under the control of either the CMV or PGK promoter. A number of transformed colonies were isolated and tested for expression of E1B-19 kDa protein by Western blot (data not shown). Ten transformed cell lines obtained by transfection with HAd5 E1 sequences under the control of CMV (FPRT-HE1-3, -4, -7, -8, -9 and -10) or PGK promoter (FPRT-HE1-1, -2, -5 and -6) were selected for further characterization. Except FPRT-HE1-1, all cell lines were isolated in the absence of G418 selection. Apart from FPRT-HE1-8 and FPRT-HE1-10, all FPRT-derived cell lines showed variable levels of E1B-55 kDa, E1A, and E1B-19 kDa expression (Fig. 2). FPRT-HE1-5 that efficiently expressed all three HAd E1 proteins was used for generation and growth of E1A-deleted PAd3 vectors. This cell line also supported plaque formation by PAd3 vectors.

3.3. Generation of PAd3 and HAd5 vectors
To generate various PAd3 recombinants [rPAd3 (wt PAd3 generated from recombinant DNA), PAd-ΔE1A, PAd-GFP, PAd-ΔE1AE3 and PAd-GFP-2], initially the full-length clones were generated by homologous recombination in bacteria and subsequently infectious vectors were produced in FPRT HE1-5 cells following transfection with full-length genomic DNA (Fig 3 A). The authenticity of all full-length clones was verified by restriction analyses (data not shown).

We also generated two replication-defective HAd5 vectors using Cre-*lox*P site-specific homologous recombination system (Ng et al., 1999). HAd-ΔE1E3 has the full-length HAd5 genome except E1 and E3 deletions, whereas HAd-GFP contained the GFP gene cassette in E1 (Fig. 3B).

3.4. Replication kinetics of PAd3 and HAd vectors in E1-expressing or E1-nonexpressing cell line
To compare replication kinetics of E1A-deleted PAd3 vectors in an E1-expressing or E1-nonexpressing cell line with that of wt PAd3, porcine E1-expressing (FPRT-HE1-5) and- E1-nonexpressing (PK-15) cell lines were infected with wt PAd3, rPAd3, PAd-ΔE1A, PAd-GFP, PAd-ΔE1AE3, PAd-GFP2, HAd-GFP, or HAd-ΔE1E3 for single-step growth curves. The wt PAd3 and rPAd3 replicated to similar titers in FPRT-HE1-5 and PK-15 cell lines (Figs. 4A and 4B). PAd-ΔE1A, PAd-GFP, PAd-ΔE1AE3, PAd-GFP2, replicated to similar titers in FPRT-HE1-5 reaching close to wt PAd3 titers at 48 h post-infection (Fig. 4A). Both HAd-GFP and HAd-ΔE1E3 replicated efficiently in FPRT-HE1-5 cells (Fig. 4C). As expected, PK-15 cells did not appear to support the replication of E1A-deleted PAd3 vectors (Fig. 4B) or E1-deleted HAd vectors (Fig. 4D).

3.5. GFP expression by PAd-GFP or HAd-GFP in infected cells
To determine GFP expression by PAd-GFP or HAd-GFP, ST cells were mock-infected or infected either with PAd-ΔE1AE3, PAd-GFP, HAd-ΔE1E3 or HAd-GFP and at 24 h post-infection cells were examined for GFP expression by fluorescence microscopy. Mock-infected cells showed only background fluorescence, whereas, cells infected with PAd-GFP, or HAd-GFP displayed extensive green fluorescence attributed to GFP expression following infection (Fig. 5A). To further confirm GFP expression in cells infected with either PAd-GFP or HAd-GFP, vector-infected cell extracts were analyzed by Western blot using a GFP-specific polyclonal antibody. HAd-GFP or PAd-GFP infected cells showed specific 27 kDa band corresponding to GFP, while mock-, PAd-ΔE1AE3-, or HAd-ΔE1E3-infected cells did not yield any specific band (Fig. 5B).

3.6. Transduction efficiency of PAd-GFP or HAd-GFP in human and murine cell lines
To compare transduction efficiencies of PAd-GFP with those obtained with HAd-GFP, we selected various cell lines of human [293, MDA-MB-231, and MCF-10A] or murine [NIH 3T3 and MTIA2] origin. The transduction efficiencies of PAd3-GFP-infected human cell lines (293, MDA-MB-231 and MCF-10A) were comparable to those obtained with HAd-GFP (Fig. 6). In addition, transduction

efficiency of PAd-GFP in murine MT1A2 cells was similar to that achieved with HAd-GFP (Fig. 6). The transduction efficiencies of HAd-GFP and PAd-GFP in murine NIH 3T3 cells were approximately 22 and 90%, respectively (Fig. 6) suggesting that PAd-GFP transduced these cells significantly (P <0.05) better than HAd-GFP.

3.7. Coxsackievirus adenovirus receptor (CAR) expression in murine and human cells

CAR is the primary receptor for group B adenoviruses including HAd5 and is involved in a high-affinity interaction with adenovirus fiber protein present on the virion surface (Bergelson et al., 1997). To determine whether there is a correlation between CAR expression and transduction efficiency of HAd or PAd vector, we examined CAR expression in murine (MTIA2 and NIH 3T3) and human (293) cell lines by Western blot and immunofluorescence using a CAR-specific antibody. High, intermediate and undetectable levels of CAR expression were observed in 293, MTIA2, and NIH 3T3 cells, respectively (Fig. 7) suggesting that transduction efficiency of PAd-GFP is not affected by the level of CAR expression.

4. DISCUSSION

Although HAd5- or PAd3-specific neutralizing antibodies were not cross-neutralizing (Moffatt et al., 2000), it was unknown whether the preexisting adenoviral neutralizing antibody response in human would cross-neutralize PAd3. In the present study, the results of virus neutralization experiments using randomly collected human serum samples clearly suggested that the majority of human population have varying degree of HAd-neutralizing immunity confirming a number of previous studies(Chirmule et al., 2000; Hackett et al., 2000; Kremer et al., 2000). More importantly, none of the serum samples neutralized PAd3 suggesting that preexisting immunity against PAd3 is not prevalent in human population. Nonhuman adenoviruses, including PAd3, are expected to be less prevalent in human population and their use in humans could efficiently circumvent preexisting immunity to HAd. It has been shown that human sera samples that neutralize HAd5 did not cross neutralize canine adenovirus type 2 (Kremer et al., 2000), ovine adenovirus type 7 (Hoffmann et al., 1999) or simian adenovirus (Farina et al., 2001). It is expected that similar to HAd vectors, the first use of nonhuman adenoviral vectors in humans will also result in vector-specific immunity. Therefore, the primary application of these vectors will be in sequential administration of two or more adenoviral vectors where readministration is necessary for the desired therapeutic effect.

For generating replication-defective (E1A-deleted) PAd3 vectors, we isolated FPRT HE1-5 cell line that expresses HAd5 E1 proteins and supports the replication of wt as well as E1A-deleted PAd3 or E1-deleted HAd5 vectors. Compared to VIDO R1, a similar cell line of porcine origin (Reddy et al., 1999b), FPRT HE1-5 was isolated without G418 selection and thus is likely to be stably transformed. Recently another trans-complementing cell line of porcine origin was produced that expresses E1B[large] protein of PAd3 and thus supports replication of E1-deleted PAd3 vectors (Zakhartchouk et al., 2003). Since we used FPRT HE-1-5 to grow only E1A-deleted PAd3 vectors, it is not known whether this cell line will also support the replication of E1A- and E1B-deleted PAd3 vectors.

In addition to the circumvention of neutralizing antibody response against HAd vectors, the other important factor in determining the usefulness of PAd3 vectors for gene therapy would be their ability to efficiently transduce human cells. Since the majority of preclinical evaluation of various adenoviral vectors are conducted in mouse models, it is equally important that these vectors also transduce murine cells with a high efficiency.

Our results of transduction experiments in human (293, MDA-MB-231, and MCF-10A) or murine (MT1A2) cells demonstrated that PAd-GFP transduced these cell lines with comparable or better

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efficiency than HAd-GFP. PAd3 vectors have also been reported to efficiently transduce human osteosarcoma (SAOS-2) and cervical carcinoma (HeLa) cell lines (Zakhartchouk et al., 2003), thereby suggesting a potential application of these vectors in cancer gene therapy.

Interestingly, we observed striking differences in transduction efficiencies of HAd-GFP and PAd-GFP in murine NIH 3T3 cells. PAd-GFP transduced NIH 3T3 cells significantly better than HAd-GFP. This difference could possibly be attributed to the tropism of HAd5 vectors that is primarily determined by the distribution of CAR on the cell surface. Entry of HAd5 into host cells is mediated by the initial binding of adenoviral fiber knob to CAR (Bergelson et al., 1997) and subsequent interactions with the secondary receptors (Arnberg et al., 2000; Hong et al., 1997) leading to the receptor-mediated endocytosis. Reduced expression of CAR and other cell surface molecules, which are necessary for HAd internalization, on certain human cell types including a variety of cancers (Jee et al., 2002; Li et al., 1999) poses a potential limitation to HAd vector-based gene therapy.

To avoid preexisting HAd immunity, HAd35 was developed as an expression vector (Reddy et al., 2003). Since HAd35 cell entry is mediated by CD46 (Gaggar et al., 2003), it did not efficiently transduce mouse cells in vivo suggesting that the majority of mouse tissues do not efficiently express CD46. Although chimpanzee adenovirus also circumvented HAd-specific neutralizing immunity, virus internalization into susceptible cells was CAR-mediated (Cohen et al., 2002). The role of CAR in canine adenovirus-2 internalization is not clear (Soudais et al., 2000).

To determine the reason for variability in transduction efficiencies of HAd-GFP in MT1A2 and NIH 3T3 cells, we examined the levels of CAR expression in these cells and found that MT1A2 efficiently expressed CAR, whereas NIH 3T3 were CAR-deficient. This was consistent with earlier observations (Fontana et al., 2003; Jee et al., 2002) indicating that CAR expression is important for efficient transduction with HAd5 vectors. Receptors that mediate PAd3 entry into susceptible cells are currently unknown; however, amino acid residues of the HAd5 fiber knob, which are known to be critical for CAR binding (Roelvink et al., 1998), are not conserved in the PAd3 fiber. Furthermore, the integrin-binding RGD motif present in the penton base protein of HAd5 is absent in PAd3 (Reddy et al., 1998). Since PAd-GFP efficiently transduced both MT1A2 and NIH 3T3 cells, it appeared that internalization of PAd3 into susceptible cells was CAR-independent. Further investigation is required to confirm this observation.

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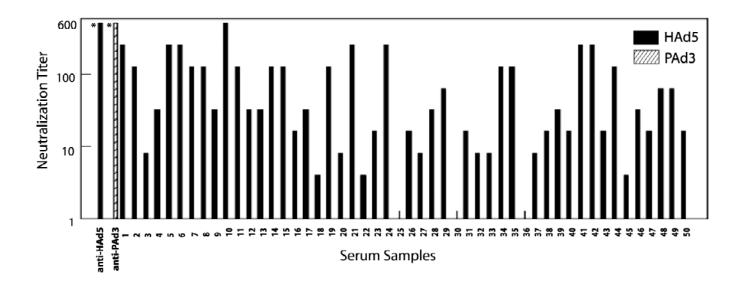
Fig. 1. Detection of preexisting HAd5-neutralizing and PAd3 cross-neutralizing antibodies in human serum samples. A total of 50 randomly collected serum samples from healthy individuals were subjected to virus neutralization tests against HAd5 and PAd3. Neutralization titer is the reciprocal of highest serum dilution that prevented viral c.p.e.

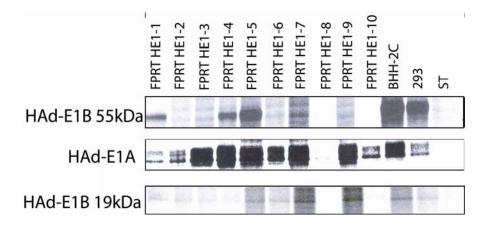[* The anti-HAd5 hyperimmune serum and anti-Pad3 hyperimmune serum served as positive control for HAd5 and PAd3 virus neutralization, respectively]

Fig. 2. Expression of adenoviral E1 proteins by fetal porcine retinal (FPRT) cells transformed with HAd5 E1. Ten E1-transformed clones (FPRT HE1-1 to 10), 293, bovine-human hybrid (BHH-2C) and ST cells were metabolically labeled with Trans [^{35}S]-label containing methionine and cysteine. Cell lysates were immunoprecipitated using for antibodies specific to adenoviral E1A (HAd2 E1A) and E1B (HAd5 E1B-19kDa and HAd5 E1B-55kDa). Immunoprecipitated proteins were resolved by SDS-PAGE and the gels were analyzed by autoradiography.
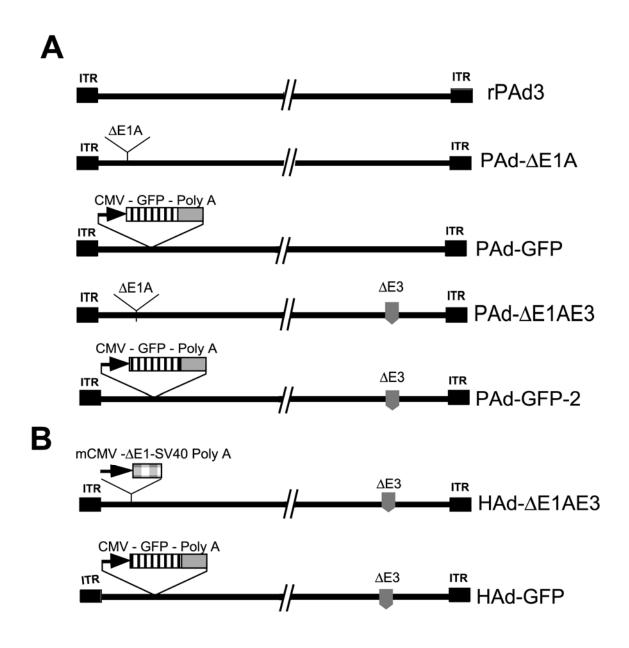
Fig. 3. Diagrammatic representation of structures of PAd3 and HAd5 vectors. (A) PAd3 vectors: PAd-ΔE1A (PAd3 vector containing 695 bp deletion in E1A region), PAd-GFP (PAd-ΔE1A containing GFP gene under the control of the CMV promoter and BGH Poly A in E1A), PAd-ΔE1AE3 (PAd-ΔE1A with an additional 600 bp deletion in E3), PAd-GFP-2 (PAd-GFP with an additional 600 bp deletion in E3); (B) HAd5 vectors: HAd-ΔE1E3 (HAd5 with E1 and E3 deletions), HAd-GFP (HAd-ΔE1E3 with the GFP gene under the control of CMV promoter and BGH Poly A in E1). ITR, inverted terminal repeat; CMV, human cytomegalovirus immediate early promoter; mCMV, mouse cytomegalovirus immediate early promoter; Poly A, bovine growth hormone polyadenylation signal; SV40 Poly A, simian virus 40 polyadenylation signal; ΔE1, deletion in early region 1 (E1), ΔE3, deletion in early region 3 (E3).
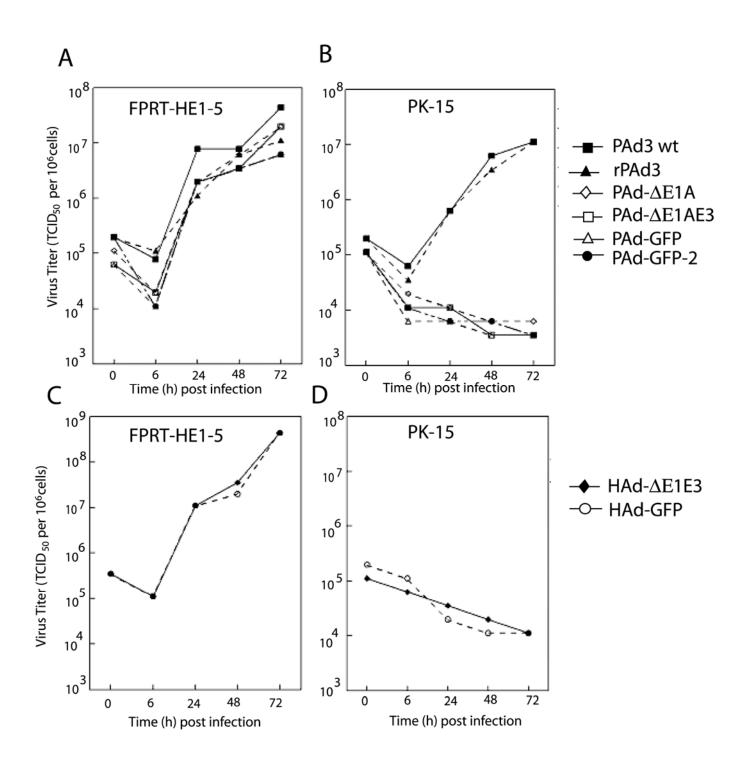
Fig. 4. Replication kinetics of PAd3 and HAd5 vectors in E1-complementing and E1-noncomplementing cells of porcine origin. FPRT-HE1-5 (E1-complementing) and PK-15 (E1-noncomplementing) cells grown to approximately 90% confluency in 60 mm dishes were infected with wt PAd3, rPAd3, PAd-ΔE1A, PAd-GFP, PAd-ΔE1AE3, PAd-GFP-2, HAd-ΔE1E3, or HAd-GFP at an m.o.i. of 0.2-0.6 $TCID_{50}$./cell. The virus-infected cells harvested with supernatant at 0, 6, 24, 48 and 72 h post-infection and subjected to three freeze-thaw cycles. PAd3 and HAd5 vectors were titrated on FPRT-HE1-5 or BHH-2C cells, respectively for determining $TCID_{50}$ (50% tissue culture infective dose). The viral titers are expressed as $TCID_{50}$ per 10^6 cells. (A) PAd3 vectors in FPRT HE1-5 cell line, (B) PAd3 vectors in PK-15 cell line, (C) HAd5 vectors in FPRT HE1-5 cell line, and (D) HAd5 vectors in PK-15 cell line.
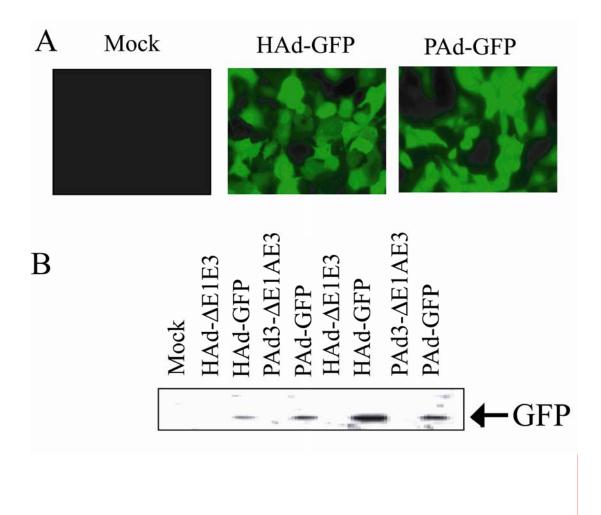
Fig. 5. Transgene expression by PAd-GFP and HAd-GFP. (A) ST cells grown on coverslips were mock-infected or infected with HAd-ΔE1E3, HAd-GFP, PAd-ΔE1AE3 or PAd-GFP at an m.o.i. of 10 p.f.u./cell. At 24 h post-infection, the coverslips were washed with PBS, fixed and observed under fluorescence microscope to observe GFP fluorescence. Mock-infected, HAd-ΔE1E3-, or PAd-ΔE1AE3-infected cells served as negative controls. (B) ST cells in monolayer cultures were infected with HAd-ΔE1E3, HAd-GFP, PAd-ΔE1AE3, or PAd-GFP at an m.o.i. of 10 p.f.u./cell. The cells were harvested at 24 and 48 h post-infection and cell lysates subjected to SDS-PAGE followed by immunoblotting with a chicken polyclonal GFP-specific antibody and a goat anti-chicken HRP-conjugated antibody.
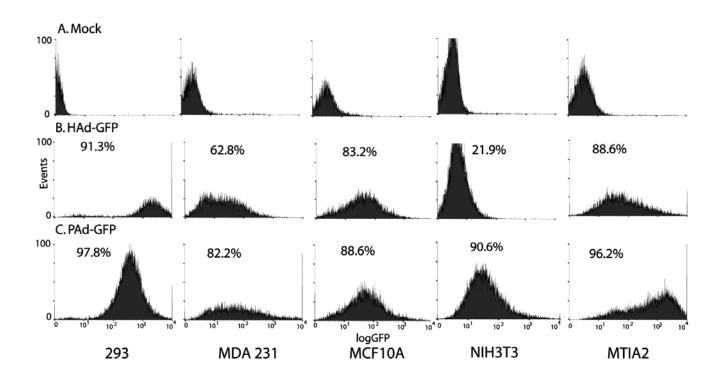
Fig. 6. Transduction efficiencies of PAd-GFP and HAd-GFP in human and murine cell lines. Cells grown as monolayers in 6-well plates were mock-infected or infected with PAd-GFP or HAd-GFP at an m.o.i. of 10 p.f.u. per cell. At 24 h post-infection, cells were harvested by trypsinization and GFP transduced cells were sorted by flow cytometry. Mock infected cells served as negative controls.
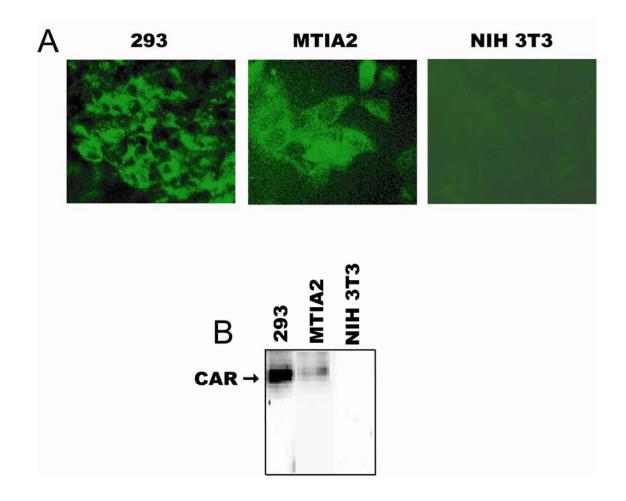
Fig. 7. CAR expression in human and murine cell lines. (A) Human (293) and murine (MTIA2 and NIH 3T3) cells grown in coverslip cultures were processed for immunofluorescence using a rabbit polyclonal anti-human CAR antibody and a FITC-conjugated goat anti-rabbit IgG. Cells were visualized under a fluorescence microscope and photographed at ×400. (B) Cell lysates of 293, MTIA2, and NIH 3T3 were analyzed for Western blot using a rabbit polyclonal anti-human CAR antibody and a HRP-conjugated anti-rabbit IgG. The signal was detected by chemiluminescence.