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Persistence and the state of bovine and porcine adenoviral vector genomes in human and nonhuman cell lines

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Abstract

The state of vector genome in transduced cells influences the duration of transgene expression and can be a safety concern if it gets integrated randomly into the host genome. Although human adenovirus (Ad) serotype 5 (HAd5) mainly persists in a linear episomal form, information regarding the state of bovine Ad serotype 3 (BAd3) and porcine Ad serotype 3 (PAd3) vector genomes in human and nonhuman cells is currently unknown. To address this issue, MDA-MB-231 (human), MDBK (bovine), PK-15 (porcine), MT1A2 (mouse) and NIH-3T3 (mouse) cell lines were infected with replication-defective BAd3, PAd3 or HAd5 vectors carrying the green fluorescent protein (GFP) gene. The persistence and the state of vector genome were assessed by quantitative real-time PCR and Southern blot hybridization, respectively. Levels of transgene and Ad gene expressions were quantified using real-time RT-PCR. Persistence of BAd3 or PAd3 vectors was comparable to that of HAd5 vector. Only the linear episomal form of the vector genome was observed with each vector. In addition, expression levels of transgene as well as viral genes by all three vectors were comparable and correlated with their transduction levels in each cell type. These results indicate comparable biologic behavior of BAd3, PAd3 and HAd5 vectors in cell culture.

Keywords

adenovirus persistence; bovine adenovirus; gene therapy; nonhuman adenovirus; porcine adenovirus; vector safety

1. INTRODUCTION

Adenoviral (Ad) vectors have demonstrated tremendous potential as a gene delivery vehicle for vaccine and gene therapy applications (Sharma et al., 2009b; Vemula and Mittal, 2010). Although human Ad (HAd) serotype 5 (HAd5) is the prototype Ad vector that has been widely used for gene delivery, several concerns hamper its widespread clinical application. HAd5 are ubiquitous in the human population and often cause self-limiting respiratory or gastrointestinal tract infections, but can also be lethal in infants and immunocompromised individuals. More than 90% of the adult population has detectable levels of circulating

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neutralizing antibodies against one or more HAd serotypes. This pre-existing vector immunity against HAdS can potentially neutralize the therapeutic vector compromising its desired efficacy. Furthermore, prominent hepatotropism, lack of specific targeting, possible recombination of a replication-defective vector with the wild type Ad to produce replication-competent Ad, long-lasting HAd-specific memory CD4⁺ T cells, and acute *in vivo* toxicity (at very high vector doses) are other potential limitations of HAd vectors. Some of these limitations could be circumvented with the use of vectors based on less prevalent HAd serotypes such as HAd3, HAd11, and HAd35 or nonhuman Ads such as bovine Ad (BAd), porcine Ad (PAd), ovine Ad, canine Ad, simian Ad, and fowl Ad (Bangari and Mittal, 2006b; Stone and Lieber, 2006). Vectors based on nonhuman Ads or chimeric HAd vectors carrying the knob or entire fiber from nonhuman Ads demonstrate novel and expanded tropism, in addition to evading HAd-specific immunity (Bangari and Mittal, 2006a; Glasgow et al., 2004).

Earlier we demonstrated that nonhuman Ad vectors based on BAd serotype 3 (BAd3) and PAd serotype 3 (PAd3) efficiently transduced several types of human and murine cells in culture (Bangari et al., 2005a). These vectors appeared to utilize distinct receptors for cell internalization (Bangari and Mittal, 2005; Bangari et al., 2005b), and cell entry was independent of the Coxsackie virus-adenovirus receptor (CAR), the primary HAd5 receptor. Most importantly, we demonstrated that there were no pre-existing virus neutralizing antibodies against PAd3 or BAd3 in humans, and HAd5-neutralizing antibodies did not cross-neutralize PAd3 or BAd3 (Bangari et al., 2005a; Moffatt et al., 2000). Furthermore, at the level of cell-mediated immunity, no significant cross-reactivity among HAd5, BAd3 and PAd3 was observed (Sharma et al., 2010). All these observations support the rationale for the use of vectors derived from BAd3 and PAd3 as supplements and/or alternatives to HAd vectors.

In the present study, we assessed the safety aspect of replication-defective BAd3 and PAd3 vectors carrying the green fluorescent protein (GFP) gene as a reporter in cell lines of human, porcine, bovine and murine origin. We compared vector persistence, state of vector genome, transgene and vector gene expression by PAd3, BAd3, and HAd5 vectors. This study demonstrates that with respect to these parameters, the safety aspects of BAd3 and PAd3 vectors are similar to that of HAd5 vectors.

2. MATERIALS AND METHODS

2.1 Ad vectors and cell lines

Replication-defective HAd-GFP (Bangari and Mittal, 2004), PAd-GFP (Bangari and Mittal, 2004) and BAd-GFP (Bangari et al., 2005a) vectors with deletions in E1 or E1A region and carrying the GFP gene under the control of the cytomegalovirus (CMV) promoter were propagated as described previously. HAd-GFP, PAd-GFP and BAd-GFP were grown and titrated in 293 (human embryonic kidney cells expressing Ad E1) (Graham et al., 1977), FPRT HE1-5 (fetal porcine retina cells expressing Ad E1) (Bangari and Mittal, 2004) and FBRT HE1 (fetal bovine retina cells expressing Ad E1) (van Olphen et al., 2002), respectively. The virus purification was done by cesium chloride-density gradient centrifugation as previously described (Bangari and Mittal, 2004). The vector titration was done by plaque assay (Bangari and Mittal, 2005).

MDA-MB-231 (human), PK-15 (porcine), MDBK (bovine), MT1A2 (murine) and NIH-3T3 (murine) cell lines were propagated as described previously (Bangari et al., 2005a). Each cell line was seeded in 150 mm tissue culture plates and, twenty-four hours later, were infected either with HAd-GFP, BAd-GFP, or PAd-GFP at a multiplicity of infection (m.o.i.) of 10 plaque forming units (p.f.u.) per cell. After 45 min of adsorption at 37 °C, the cells

were supplemented with minimum essential medium containing 2% fetal calf serum. Cell lines were maintained for a period of 24 days (d) by passing them every fourth day with a split ratio of 1:3. At 2 h, 12 h, 1 d, 2 d, 4 d, 8 d, 16 d and 24 d post-infection, the cells were harvested and processed for RNA or DNA isolation. At each time point, the cells were counted, and the data were adjusted to reflect the cell number at the time of infection.

2.2 Primers and Taqman probes

A primer pair and a Taqman® probe specific to the GFP, E4 (HAD5 E4 34 kDa orf, PAD3 E4 orf 8 and BAD3 E4 orf 3) or hexon genes of HAd-GFP, PAD-GFP, or BAD-GFP were designed using Primer Express 2.0 software (Applied Biosystems, Foster city, CA) (Sharma et al., 2009a). These primers and probe were used for quantification of vector genomes by real-time PCR, and for analyzing GFP, E4 or hexon expression by real-time RT-PCR. All primer sets and Taqman® probes were synthesized by Applied Biosystems. All probes were labeled with 6-carboxyfluorescein (FAM) at the 5' end and with minor groove binder (MGB) at the 3' end.

2.3 Quantification of vector genome

Total genomic DNA from vector-infected cells was isolated using DNeasy kit (Qiagen Inc., Valencia, CA) as per the manufacturer's guidelines. Initially, standard curves were generated using 10-fold dilutions (from 3 to 3×10^8 copies) of purified genomic DNA of HAd-GFP, PAD-GFP, or BAD-GFP as a template for real-time PCR using GFP primers and the probe. The copy number of each vector genome was calculated based on spectrophotometric quantification and the molecular mass of vector genomic DNA. Subsequently, various dilutions of each vector were run as standards with each set of real-time PCR assay. DNA samples from mock-inoculated cells served as negative controls. For quantification of the vector genome by real-time PCR using the GFP primers and probe, 50 ng of the total cellular DNA was used in a 25 µl reaction using Taqman® PCR core reagents (Applied Biosystems) as described (Sharma et al., 2009a). The threshold cycle (Ct) value for individual reactions was determined, and data were analyzed with MxPro software (Stratagene) to obtain the absolute copy number of viral genome per 50 ng of total cellular DNA. The copy numbers of the vector genome for each time point were adjusted to reflect the cell number at the time of infection.

2.4 Quantification of GFP, E4 or hexon mRNA transcripts

Total cellular RNA was isolated from vector-infected cells using RNA miniprep kit (Stratagene). RNA samples were treated with DNase I to remove the residual DNA. Two hundred ng of total cellular RNA was processed for real-time RT-PCR using GFP-, E4-, or hexon-specific primers and probe and One-step Brilliant QRT-PCR Master Mix Kit (Stratagene) as described (Sharma et al., 2009a). The Ct values for individual reactions were determined, and data were analyzed with MxPro software for absolute quantification of GFP, E4, or hexon mRNA transcripts. All RNA samples were also used for PCR assay using GFP, E4, or hexon primers and probe to ensure the absence of the residual DNA. The copy numbers of the mRNA transcripts for each time point were adjusted to reflect the cell number at the time of infection.

2.5 Southern blot analysis

Total cellular DNA from vector-infected cell lines was isolated by proteinase-K digestion and the isopropanol precipitation method (Sambrook J and Russell D.W., 2001). Twenty µg of the total cellular DNA was digested with *KpnI* (for HAd-GFP infected cells), *XhoI* (for PAD-GFP infected cells) or *HpaI* (for BAD-GFP infected cells) and loaded onto a 1% agarose gel and processed for Southern blot analysis as described (Sharma et al., 2009a).

DNA hybridization was performed using HAd-GFP (nt 34,524–35,537), PAd-GFP (nt 32,353–33,582) or BAd-GFP (nt 32,520–33,615) specific probes which were selected from the area near the right terminus of the vector genome to assist in identification of the linear, circular, or integrated form of the vector genome. The rest of the protocol was similar as described earlier (Sharma et al., 2009a). The blots were exposed to Cyclone Storage Phosphor Screen (Packard Instrument Company, Meriden, CT), and signals were visualized by Cyclone Storage Phosphor System (Packard Instrument Company).

3. RESULTS

3.1 Vector persistence in cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP

To investigate whether PAd3 and BAd3 vectors have persistence significantly different from that of HAd5, non-E1-complementing cell lines of human (MDA-MB-231), porcine (PK-15), bovine (MDBK) or murine (MT1A2 and NIH-3T3) origin were infected with HAd-GFP, PAd-GFP, or BAd-GFP at the m.o.i. of 10 p.f.u. Cell lines were maintained by passing them every fourth day, and no cytopathic effect was observed in any of the cell lines infected with any of the viruses. At 2 h, 12 h, 1 d, 2 d, 4 d, 8 d, 16 d and 24 d post-infection, the cells were harvested, and total cellular DNA extracted. The DNA samples were analyzed for quantification of vector genomes by real-time PCR specific to the GFP transgene as it was present in all three vectors. The sensitivity of this assay was three copies of the vector genome per 50 ng of total cellular DNA. In all the cell lines, the HAd-GFP, PAd-GFP or BAd-GFP vector genomes were detected as early as 2 h post-infection and persisted up to 24 d post-infection (Figs. 1A, B & C). Notably, in the absence of immune pressure, 1–2 logs reduction in the genomic levels at 24 days was observed compared to the 2 h time point in HAd-GFP, PAd-GFP, or BAd-GFP infected cells. The levels of vector genome in a cell line correlated with the previously observed transduction efficiency of the vector in that cell line (Bangari et al., 2005a). For example, in contrast to the BAd-GFP or PAd-GFP genome levels, only low levels of HAd-GFP genomes were detected in vector-infected NIH-3T3 cells because of low infectivity of HAd-GFP in this cell line. In MDA-MB-231 cell line, BAd-GFP persisted at low levels. No vector genome was detected in the mock-infected cell lines at any time point (data not shown).

3.2 State of vector genome in cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP

The HAd5 genome usually persists in an episomal form within the host cell nucleus (Ehrhardt et al., 2003; Ehrhardt et al., 2008). However, the vector genome may also get integrated into the chromosomal DNA at a very low frequency (10^{-3} to 10^{-5} per cell) (Harui et al., 1999). In order to address the safety aspect of PAd3 and BAd3 vectors, it was important to determine the state of their genomes in the cultured cells of various animal species. To assess the state of vector DNA in HAd-GFP, PAd-GFP, or BAd-GFP-infected cell lines, samples of the total cellular DNA extracted from cell lines harvested at various times post-inoculation were subjected to Southern blot analyses. The probes for Southern blot assays were designed to distinguish the linear episomal, circular episomal and integrated forms of HAd-GFP, PAd-GFP, or BAd-GFP genome based on the fragment size of restriction endonuclease (RE)-digested DNA. The sensitivity of the Southern blot assay, as determined by the amount of vector genome used as a positive control, was found to be 10 pg (approximately 2.6×10^5 copies) of vector DNA. The expected fragment sizes for three forms of DNA (linear episomal, circular episomal, and integrated) were 2.3, 3.4, and >2.3 kb for HAd-GFP, 3, 5, and >3 kb for PAd-GFP, and 2, 6.8, and >2 kb for BAd-GFP (Fig. 2A). In HAd-GFP, PAd-GFP or BAd-GFP-infected cell lines, vector-specific bands of 2.3, 3, or 2 kb, respectively, were detected up to 8 d post-infection suggesting that only the linear forms of the vector genomes were present in HAd-GFP, PAd-GFP, or BAd-GFP infected cell lines (Figs. 2B, C & D). Bands of DNA that would correspond to a circular or

integrated form of the vector genome were not observed even after overexposure of the Southern blots (data not shown). No signals were detected in samples from mock-infected cell lines (data not shown). The presence of a circular form of genomes was also investigated by PCR using specific primer sets for each vector, but none of the tissue samples yielded positive results (data not shown). Sensitivity of PCR assay for detection of the circular form of HAd5 genome was determined by using serial dilutions of a plasmid (pDC311; Microbix Biosystems, Toronto, ON, Canada) that contained HAd5 end fragments joined together, was 2.5 pg or 7×10^5 copies (data not shown). It seemed that Southern blot was approximately 2.6-fold more sensitive compared to PCR assay. The suboptimal sensitivity of PCR could be due to the presence of the two long inverted terminal repeats in these Ad vector genomes and the GC-rich nature of the template resulting in complex secondary structures. Nevertheless, the results of Southern blot analyses and PCR indicated that the majority of HAd-GFP, PAd-GFP or BAd-GFP genomes remained as linear episomes.

3.3 Levels and duration of transgene expression in various cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP

Efficacy of any gene delivery vector largely depends on the level and duration of transgene expression. In order to further evaluate the efficiency of these vectors, the total cellular RNA from various cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP were quantitated for GFP-specific mRNA transcript levels by real-time RT-PCR. Expression of GFP transgene was observed as early as 2 h post-infection in all the cell lines and gradually peaked by 1 or 2 d post-infection followed by a gradual decline (Figs. 3A, B & C). The transgene expression persisted for up to 16 or 24 d post-infection. In general, the efficiency of transgene expression by HAd-GFP, PAd-GFP or BAd-GFP was comparable and correlated with the levels of vector genomes in that particular cell line. No GFP expression was detected in mock-infected cell lines at any time point (data not shown).

3.4 Levels and duration of the early (E4) and late (hexon) gene expression in various cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP

Expression of vector genes at lower levels ("leaky gene expression") has been reported in E1-deleted replication-defective vectors (Elkon et al., 1997; Kafri et al., 1998; Yang et al., 1994). Paradoxically, minimal levels of vector gene expression is better for vector survival or persistence as it helps to shield the vector-transduced cells from the host immune surveillance (Russell, 2000; Yang et al., 1994). To investigate whether vector gene expression by nonhuman Ad vectors (BAd-GFP and PAd-GFP) would be comparable with those of HAd-GFP, the total cellular RNA from various cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP were quantitated for E4 or hexon mRNA transcript levels by real-time RT-PCR. We selected an E4 (HAd5 E4 34 kDa open reading frame (orf), PAD3 E4 orf8 and BAd3 E4 orf3) gene as a representative of an early Ad gene and the hexon gene as a representative of a late Ad gene. In all the cell lines, mRNA expressions of E4 (Figs. 4A, B & C) and hexon (Figs. 5A, B & C) were observed as early as 2 h post-infection and reached their maximum level at 12 h (E4) or 1 d (hexon) post-infection followed by a gradual decline. In all cell lines, no detectable expression of E4 or hexon was observed at 8 d post-infection or later except with BAd-GFP where expression of hexon was observed at 8 d post-infection in MDBK cell line. The expression level of both E4 and hexon by PAd-GFP or BAd-GFP were comparable to those observed by HAd-GFP and correlated with vector genome levels in each cell line. No expression of either E4 or hexon was observed in mock-infected cell lines (data not shown).

4. DISCUSSION

We have developed gene delivery vectors based on nonhuman Ad-derived from swine (PAd3) and cattle (BAd3) (Bangari and Mittal, 2004; Mittal et al., 1995). These vectors efficiently transduced a number of human and nonhuman cells in culture (Bangari et al., 2005a) and a variety of mouse tissues following systemic inoculation (Sharma et al., 2009a). In this study we characterized these vectors in terms of their persistence, the state of vector genome, and transgene and vector gene expression in a panel of cell lines to explore their safety profiles for gene delivery applications.

An ideal gene delivery vector should be able to efficiently transduce the target cells and ensure transgene expression at high levels for a sufficient duration. BAd-GFP, PAd-GFP and HAd-GFP vectors showed variable levels of vector genomes indicating differential transduction in various cell lines. Vector genome persistence in all cell lines was comparable among the three vectors. Differential transduction efficiencies of HAd5, BAd3 and PAd3 vectors in a variety of human, murine, porcine and bovine cell lines have previously been reported (Bangari et al., 2005a). In most cell lines, vector genome levels of HAd-GFP, PAd-GFP, or BAd-GFP showed good correlation with the previously reported transduction efficiency of these vectors. Variability in the transduction efficiencies of these vectors in these cell lines may be due to distinct receptor usage by these adenoviral vectors (Bangari et al., 2005a; Bangari and Mittal, 2005; Bangari et al., 2005b; Li et al., 2002). In the absence of immune pressure, HAd5, PAd3 and BAd3 vectors persisted in various cell lines at least 24 d post-infection (the last time-point in this study) in significant amounts; however, the mechanism/s of their survival is/are unknown. It is anticipated that some vector-infected cells may be lost over the period mainly due to repeated passage of various cell lines. As anticipated, transgene expression by HAd5, PAd3 and BAd3 vectors in various cell lines followed the patterns of their respective genome numbers.

Following cell entry, Ad genomes may persist as a linear episomal form, circular episomal form, or integrated into the host genome (Ehrhardt et al., 2008). It has been reported that the majority of the HAd5 genome in transduced cells persists in an episomal form (Ehrhardt et al., 2003; Harui et al., 1999). However, a fraction of the vector genome may get integrated into the host chromosomal DNA (Harui et al., 1999). It seems that the genomes of some HAd serotypes such as HAd12 have a higher frequency of random integration into the host genome (Green et al., 1977; Knoblauch et al., 1996). Although prolonged vector persistence leading to a sustained transgene expression is important for a desired therapeutic effect, random vector insertion may lead to insertional mutagenesis - a potential safety concern. The circularized vector genome may replicate to some extent leading to its enhanced copy number and persistence (Kreppel and Kochanek, 2004). In this study, only the linear episomal form of HAd-GFP, PAd-GFP, or BAd-GFP genome was detected suggesting that the majority of the PAd-GFP or BAd-GFP genome remained as a linear episome, similar to the HAd-GFP genome. In contrast to the study by Harui *et al.* (1999), our study fails to detect any integrated vector DNA in any of the cell lines. This inconsistency in the results could be because of the differences in the methods used, as Harui *et al.* used G418 selection to force integration, while we directly detected the vector DNA in the vector-infected cells. In another study, Zeng *et al.* (1997) demonstrated integration of HAd5 vector genome in irradiated NIH-3T3 cells but not in regular NIH3T3 cells infected with HAd5 vector (Zeng et al., 1997).

Proteins encoded by adenoviral early (E1) region are essential for the transactivation of other early and late genes (Russell, 2000); therefore, E1-deleted Ad vectors are replication-defective. However, leaky expression of the early and late genes of such vectors has been reported even in the absence of E1 complementation (Elkon et al., 1997; Kafri et al., 1998;

Yang et al., 1994). Unlike HAd-GFP in which the entire E1 region was deleted, only E1A regions were deleted in PAd-GFP and BAd-GFP used here (Bangari et al., 2005a; Bangari and Mittal, 2004). Partial deletion in the E1 region of PAd-GFP and BAd-GFP was found to be sufficient to render these vectors replication defective (Bangari and Mittal, 2004; Reddy et al., 1999; van Olphen et al., 2002). Despite the partial E1 deletion in PAd-GFP or BAd-GFP, expression of E4 (early) and hexon (late) genes corresponded to the amount of vector present in various cell lines, similar to that of HAd-GFP levels. Leaky vector gene expression assists in induction of vector-specific cytotoxic T lymphocyte response and is also responsible for identification and removal of vector-transduced cells by immune surveillance mechanisms. Overall, these *in vitro* data suggest that the safety and biological properties of PAd3 and BAd3 vectors are similar to those of HAd5-based vectors.

5. CONCLUSIONS

Only the linear episomal form of the HAd-GFP, PAd-GFP, or BAd-GFP genome was detected in cell lines of human, murine, bovine, or porcine origin with no evidence of the vector genome integration into the host genome. Additionally, the BAd3, PAd3, or HAd5 vector genomes persisted at high levels in vector-permissive cell lines in the absence of immune pressure. Overall, the safety and biological aspects of both PAd3 and BAd3 vectors appear similar to a HAd5 vector.

Highlights

- In the absence of immune pressure, the BAd3, PAd3, or HAd5 vector genomes persisted at high levels in various cell lines.
- Only the linear episomal form of the vector genome was observed with each vector.
- Transgene expression levels in various cell lines were correlated with the level of vector genome presence.
- Vector early or late gene expression by each vector followed the vector transduction efficacy.
- The data highlighted that the *in vitro* safety aspect of PAd3 or BAd3 vector is similar to that of HAd5 vector.

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Figure 1. Persistence of vector genome in various cell lines infected with A) HAd-GFP, B) PAd-GFP, or C) BAd-GFP

Genome concentrations are expressed as the number of vector genome copies per 50 ng of total cellular DNA quantitated by real-time PCR.

Figure 2. State of vector genome in various cell lines infected with HAd-GFP, PAd-GFP, or BAd-GFP

DNA samples from HAd-GFP, PAd-GFP, or BAd-GFP-infected cells (MT1A2, MDBK, PK-15, NIH-3T3 and MDA-231) were digested with *KpnI*, *XhoI*, or *HpaI* restriction enzymes, respectively. Similarly treated DNA samples from the mock-infected cells served as a negative control, and purified vector DNA served as a positive control. A) Depiction of experimental plan: Southern blots were performed with vector-specific probes representing the sequences near the right end of the genome indicated by the heavy black bars. The results of Southern blots showing linear-form of the vector genomes of B) HAd-GFP, C) PAd-GFP and D) BAd-GFP are presented. The lower arrow in each blot (B, C or D)

represents the expected band of the linear-form of the genome, and the upper arrow indicates the expected band of the circular form of the genome.

Figure 3. Levels of transgene mRNA expression in various cell lines infected with A) HAd-GFP, B) PAd-GFP, or C) BAd-GFP

Real-time RT-PCR for quantification of GFP mRNA was performed using 200 ng of the total cellular RNA.

Figure 4. Levels of E4 mRNA expression in various cell lines infected with A) HAAd-GFP, B) PAd-GFP, or C) BAd-GFP

Real-time RT-PCR for quantification of E4 mRNA was performed using 200 ng of the total cellular RNA.

Figure 5. Levels of hexon mRNA expression in various cell lines infected with A) HAd-GFP, B) PAd-GFP, or C) BAd-GFP
Real-time RT-PCR for quantification of hexon mRNA was performed using 200 ng of the total cellular RNA.