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Sequential administration of bovine and human adenovirus vectors to overcome vector immunity in an immunocompetent mouse model of breast cancer

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Abstract

The potential of a bovine adenovirus serotype 3 (BAd3)-based vector to bypass the human adenoviral serotype 5 (HAd5)-specific neutralizing immune response was evaluated in an immunocompetent mouse model of breast cancer. Initially we monitored vector biodistribution, genome persistence, transgene expression, and potential toxicity of HAd-GFP [HAd5 vector expressing green fluorescent protein (GFP)] or BAd-GFP (BAd3 vector expressing GFP) in FVB/n mice bearing tumors. A comparable biodistribution pattern for BAd-GFP and HAd-GFP was evident. In addition, following the development of vector-specific immune responses, animals were inoculated intratumorally (i.t.) with HAd-GFP or BAd-GFP. HAd-GFP immunity did not hamper the transduction and persistence of BAd-GFP into the tumors and other organs, and similarly, BAd-GFP immunity did not hamper the transduction and persistence of HAd-GFP. Both BAd3 and HAd5 vectors showed relatively higher transgene expression in the presence of heterologous vector immunity. In contrast, the homologous vector immunity was associated with a rapid vector clearance and decline in transgene expression levels. Histopathological changes in BAd-GFP inoculated animals were generally mild with some acute but recoverable hepatic perturbations. Overall, the data suggest the importance of BAd3 vectors for sequential vector administration in overcoming the vector immunity for cancer gene therapy.

Keywords

Bovine adenovirus vector; Breast cancer; cancer gene therapy; Vector immunity; Vector biodistribution

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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1. INTRODUCTION

Adenovirus (Ad) vectors are promising gene delivery vehicles for cancer gene therapy (Sharma et al., 2009b). While several gene therapy clinical trials are currently being pursued using human Ad (HAd) vectors (Edelstein et al., 2007), their clinical usefulness has been hampered by broad tissue tropism, strong immunogenicity and pre-existing Ad immunity in the majority of human population (Bangari and Mittal, 2006a; Chirmule et al., 1999; Schmitz et al., 1983). Strong host immune response against human Ad (HAd) vectors following the first inoculation severely limits tissue transduction after re-administration (Yang et al., 1994), and repeat administration of Ad vectors is often necessary to extend the therapeutic effect. To overcome pre-existing HAd immunity in the host, alternative gene delivery vectors for cancer gene therapy are needed.

As an alternate to HAd serotype 5 (HAd5), the most commonly used Ad vector, we have developed a bovine adenovirus (BAd3)-based vector (Bangari and Mittal, 2006a; Bangari and Mittal, 2006b). In contrast to HAd5 which utilizes the Coxsackievirus-Ad receptor (CAR) as the primary receptor for intracellular entry (Russell, 2009), the BAd3 vector is CAR-independent and uses sialic acid molecules for cell entry (Li et al., 2009). Many cancer cells have low or no CAR expression and, thus, are refractory to HAd5 transduction (Li et al., 1999; Mizuguchi and Hayakawa, 2004). However, the clinical usefulness of BAd3 vector for cancer gene therapy is currently not known.

A BAd3 vector-based vaccine for highly pathogenic avian influenza H5N1 virus showed excellent protection against lethal influenza in the presence of exceptionally high levels of HAd5 neutralizing antibodies in mice (Singh et al., 2008). The systemic inoculation of a BAd3 vector in mice revealed tissue tropism comparable to that of HAd5 with longer persistence (Sharma et al., 2009a); however, the potential of BAd3 vector-mediated transgene delivery in the context of HAd5- and transgene-specific pre-existing immunity for breast cancer gene therapy has not been explored. The objective of this study was to evaluate single or sequential i.t. administration of HAd5 and BAd3 vectors in an orthotopic mouse model of breast cancer in either the presence or absence of vector immunity. We reasoned that intratumoral (i.t.) inoculation may result in higher transgene expression locally within tumors with potential therapeutic benefits and performed a comparative analysis of biodistribution, persistence, transgene expression, and tissue toxicity following the inoculation with BAd3 and HAd5 vectors. Our results demonstrated a comparable biodistribution of BAd3 and HAd5 vectors after i.t. inoculations and the ability of the BAd3 vector to efficiently transduce tumor and systemic tissues in the presence of HAd5 and transgene immunity. The data support the potential of BAd3 vectors as an alternative or supplement to HAd5 vectors for cancer gene therapy.

2. MATERIALS AND METHODS

2.1 Cell culture and Ad vectors

The human embryonic kidney cell line (HEK 293) (Graham et al., 1977), Madin-Darby bovine kidney cell line (MDBK), bovine human hybrid cell lines (BHH3 and BHH2C) (van Olphen et al., 2002) and mammary adenocarcinoma cell line (MT1A2) (Emtage et al., 1998) were propagated as previously described (Sharma et al., 2009a). Replication-defective HAd5 and BAd3 vectors expressing the GFP gene (HAd-GFP or BAd-GFP) were developed previously and propagated in HEK 293 and BHH3 cells, respectively (Bangari et al., 2005). Purified stocks of HAd-GFP and BAd-GFP were prepared by cesium chloride density centrifugation. The Ad vector particle count (VP) was determined spectrophotometrically by measuring the absorbance at 260nm (Sharma et al., 2009a).

2.2 Animal inoculations

Six to eight week-old female FVB/n mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). Mice handling and experimentation were performed according to the guidelines of the Purdue University Biosafety Committee and Animal Care and Use Committee. The characterization of the MT1A2 cell line and the generation of an immunocompetent mouse model to study breast cancer have been previously described (Noblitt et al., 2005). Groups of mice with approximately 100 mm³ MT1A2-derived tumors were inoculated i.t. with 5×10^{11} VP of HAd-GFP or BAd-GFP. The control group was similarly inoculated with phosphate-buffered saline (PBS). The injection site was sealed with a topical tissue adhesive *Nexaband* (Abbott, Chicago, IL, USA) to avoid the seepage of vector along with the blood at the injection site. Three mice per group were sacrificed at 0.25, 0.5, 1, 2, 4, 8, and 16 days after vector inoculation, and tissue samples were collected. Representative samples of the tumor, spleen, kidney, lung, liver and heart tissue samples were snap frozen for nucleic acid isolation or preserved in 10% neutral-buffered formalin for histopathological and immunohistochemical analyses.

In another experiment, groups of mice were inoculated intramuscularly (i.m.) with 5×10^{11} VP of HAd-GFP or BAd-GFP to generate vector- and transgene-specific immune responses, while a control group was inoculated with PBS. Two weeks post i.m. inoculation, 10^6 MT1A2 cells were inoculated s.c. into the right axillary region of each mouse. Four weeks after inoculation, blood samples were collected to monitor the development of vector-specific immune response. The tumor-bearing mice with or without pre-existing vector immunity were divided into nine groups with twenty-one mice per group (three mice per time point). The control group was inoculated i.t. with PBS (group 1), 5×10^{11} VP of HAd-GFP (group 2) or BAd-GFP (group 3). The mice with pre-existing vector immunity against HAd-GFP were inoculated i.t. with PBS (group 4), 5×10^{11} VP of HAd-GFP (group 5) or BAd-GFP (group 6). Similarly the mice with pre-existing vector immunity against BAd-GFP were inoculated i.t. with PBS (group 7), 5×10^{11} VP of HAd-GFP (group 8) or BAd-GFP (group 9). Three mice per group were sacrificed at 0.25, 0.5, 1, 2, 4, 8, and 16 days after vector inoculation, and tissue samples were collected for histopathology. Representative samples of the tumor, spleen, kidney, lung, liver and heart tissue samples were snap frozen for nucleic acid isolation.

2.3 Virus neutralization assay and ELISA to detect anti-GFP immune response

The virus neutralization assay was performed according to a previously described procedure (Sharma et al., 2010b). The reciprocal of the highest serum dilution that prevented the development of cytopathic effects in cells was determined as the virus neutralization titer. The ELISA procedure to detect anti-GFP antibodies was performed using a previously described method (Sharma et al., 2009a). The absorbance was spectrophotometrically determined at 450nm. The reciprocal of highest test serum dilution with absorbance above control (mean $\pm$ 2 standard deviation) was judged as the antibody titer.

2.4 Nucleic acid isolation

Total genomic DNA was isolated from approximately 50 mg of the tumor, spleen, kidney, lung, liver, and heart tissues with DNeasy tissue kit (*Qiagen Inc.*, Valencia, CA, USA) as per the manufacturer's instruction. RNA was isolated and rendered DNA-free by using the column-based RNA isolation method as per the manufacturer's guidelines (*Stratagene Inc.*, La Jolla, CA, USA). DNA and RNA yields were quantified spectrophotometrically at 260nm.

2.5 Quantitative real-time PCR (qPCR) and quantitative reverse transcriptase real-time PCR (qRT-PCR)

Quantitative PCR was performed using TaqMan probes (*Applied Biosystems*, Foster City, CA, USA) as previously described for the early region 4 (E4) of the HAd5 or BAd3 (Sharma et al., 2009a). The vector genome was quantified by the standard curve method of absolute quantification, and the data was analyzed by MxPro software (*Stratagene Inc.*). To determine levels of the residual vector genome from the first inoculation in the repeat vector inoculation groups, two groups of mice that received PBS (group 4 and 7) as the second inoculation were used. The amount of vector genome in the corresponding PBS-inoculated mice was subtracted from the repeat inoculations groups (group 5 minus group 4; group 9 minus group 7) to determine the actual copy number from the second inoculation.

For gene expression analysis, total cellular RNA was used in qRT-PCR with GFP-specific primers and probes (Sharma et al., 2009a). A probe against 18S RNA was utilized for data normalization, and the relative mRNA expression level was determined by $\Delta\Delta C_t$ method with the Day 0.25 HAd-GFP-inoculated mouse sample as a calibrator. The mean GFP expression levels in tissue samples from HAd-GFP-inoculated animals were assigned as 1000 arbitrary units, and expression levels in other samples were calculated in relation to the Day 0.25 samples. For repeat vector inoculations, RNA expression was quantified and calibrated with respect to the residual gene expression (from the first inoculation) present in the Day 0.25 PBS-inoculated control samples of the repeat inoculation groups. The gene expression pattern of chemokines, CCL1–5, CXCL10, CXCL11, Toll-like receptors (TLR) 1–9, TICAM, and MyD88 as indicators of an innate immune response, were determined from tumor and spleen RNA samples collected at Day 0.25 and quantified as previously described (Sharma et al., 2010a). The gene expression levels of each chemokine or TLR molecule from HAd-GFP- or BAd-GFP-treated mice was calibrated with respect to the control levels (as 100%) in the PBS-inoculated mice.

2.6 Hepatic enzymes, histopathology and immunohistochemistry

Blood samples collected at Days 0.25, 1 and 2 post-vector inoculations were used to monitor the levels of hepatic enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) with previously described method (Sharma et al., 2010a).

For histopathological analysis, tissue sections were stained with hematoxylin and eosin, examined and graded for histopathological alterations by a board-certified pathologist (DSB). The immunohistochemical analysis was performed for GFP expression in tumor tissues using a mouse anti-GFP antibody (*Millipore Corporation*, Billerica, CA, USA). The analysis for Kupffer cells in the liver tissues was done using a mouse anti-F4/80 antibody (*Abcam*, Cambridge, MA, USA) (Sharma et al., 2010a).

2.7 Statistical analysis

The statistical analysis was performed using *STATA* (College Station, TX, USA) data analysis and statistics software. The genomic DNA copy number and gene expression data were logarithmically transformed and tested for significance with unpaired t-test with unequal variances. A *P* value of less than 0.05 was considered significant.

3. RESULTS

3.1 Vector biodistribution, persistence and transgene expression following i.t. inoculations with BAd-GFP or HAd-GFP in the absence of vector immunity

To evaluate the usefulness of a BAd3 vector for cancer gene therapy, tumor-bearing FVB/n mice were inoculated i.t. with either BAd-GFP or HAd-GFP. Total genomic DNA was

isolated from tumor, spleen, kidney, liver, lung, and heart samples collected at various time points (Days 0.25 to 16). Vector copy numbers were determined by qPCR using vector-specific TaqMan probes for the E4 region. In tumor tissues, the highest level of BAd-GFP genomic DNA (8.25×10^6 copies) was detectable on Day 0.25 post-inoculation and gradually declined with time, but 1.16×10^3 vector copies were still detected on Day 16 post vector inoculation (Fig. 1a). The BAd-GFP vector was also detected in the spleen, kidney, lung, liver and heart (Fig. 1b - f) but at about 10 to 1000-fold lower levels than in tumor tissues. Higher numbers of BAd-GFP genome copies were present in the spleen, kidney and heart as compared to vector copy numbers with HAd-GFP. However, vector copy numbers of both vectors were similar in the liver and lung at several time points. The results indicate that following i.t. delivery, BAd-GFP persisted in the tumor tissue for at least sixteen days and was also distributed to distant organs.

GFP expression levels in various tissue samples from mice inoculated with either BAd-GFP or HAd-GFP were determined by qRT-PCR analysis. Both BAd-GFP and HAd-GFP efficiently expressed GFP in the tumor and other organs. In mice inoculated with BAd-GFP, the highest levels of GFP expression was observed within the tumor tissue from Day 0.25 to Day 1 after vector inoculation. These levels were followed by a gradual decline over time (Fig. 2a). GFP expression levels were higher in the kidney, liver, lung, and heart of HAd-GFP-inoculated mice for the majority of time points as compared to those of BAd-GFP-inoculated mice (Fig. 2c-f).

Immunohistochemical analyses showed strong cytoplasmic GFP immunoreactivity at Day 0.25 post vector inoculation in the tumor or liver sections from mice inoculated with BAd-GFP or HAd-GFP (Fig. 3). With both BAd-GFP and HAd-GFP, GFP expression in the liver was evident mainly in scattered hepatocytes as compared to the widespread expression in the tumor tissues.

3.2 Evaluation of innate immunity and toxicity after i.t. inoculations with BAd-GFP or HAd-GFP in the absence of vector immunity

To evaluate innate immune responses following the i.t. inoculation of BAd-GFP or HAd-GFP, expression levels of pro-inflammatory chemokines, TLRs and TLR-adaptor molecules in the tumor tissue and spleen were evaluated at early time points. The gene expression pattern of a panel of chemokines was comparable in the tissues from BAd-GFP- or HAd-GFP-inoculated mice at Day 0.25 with the exception of the levels of the chemokine CCL4 which were significantly higher in the spleen and tumor tissue of the BAd-GFP inoculated group (Fig. 4a, c). With both the HAd and BAd samples, the differences in TLR expression were not appreciable with the exception of TLR1 whose levels with both vectors were over five-times lower in the tumor tissue as compared to the spleen tissue (Fig. 4b, d). In the spleen, the levels of TLR3 were about twenty times higher and the levels of adapter molecule MyD88 were about five times higher compared to the PBS-inoculated control mice (Fig. 4d).

The vector toxicity was evaluated by monitoring the Kupffer cells (KCs) number in the liver, the levels of AST and ALT in the plasma samples, and the histopathology of various tissues. In the HAd-GFP-inoculated mice livers, the immunohistochemical examination of KCs number at Days 0.25 and 0.5 revealed a significant ($P < 0.05$) decline at Day 0.25 post vector inoculation (Fig. 4g, h). The changes in KCs number in the BAd-GFP inoculated mice livers were insignificant. Significant fluctuations of the AST and ALT levels in the blood of mice were noted; however, the levels returned to PBS-inoculated control mice levels by Day 2 post-inoculation (Fig. 4e, f).

3.3 Vector biodistribution, persistence and transgene expression following i.t. inoculations with BAd-GFP or HAd-GFP in the presence of vector immunity

To establish Ad vector immunity, mice were inoculated with 5×10^{10} VP of HAd-GFP or BAd-GFP via the i.m. route. Vector-specific neutralizing antibody responses were confirmed at four weeks post vector inoculation by *in vitro* virus neutralization assays. Virus neutralization titers of 4267 ± 1847 (average $\pm$ standard deviation) and 5333 ± 1847 were observed in serum samples obtained from mice inoculated with either HAd-GFP or BAd-GFP, respectively. In addition, anti-GFP ELISA titers of 12,800 and 6,400 were observed in serum samples obtained from mice inoculated with either HAd-GFP or BAd-GFP, respectively. To determine the effects of vector immunity on vector biodistribution, persistence and transgene expression, the tumor-bearing FVB/n mice having pre-existing Ad vector immunity, were inoculated with HAd-GFP, BAd-GFP or PBS.

In the tumor tissues, the presence of homologous (HAd-GFP vs. HAd-GFP; BAd-GFP vs. BAd-GFP) or heterologous (HAd-GFP vs. BAd-GFP; BAd-GFP vs. HAd-GFP) vector immunity did not significantly affect the vector persistence within the tumor tissues with the exception of the clearance of BAd-GFP at Day 8 and Day 16 post-inoculation in the presence of a homologous vector immune responses (Fig. 5a, b). This result suggests that both BAd-GFP and HAd-GFP efficiently transduced the tumor tissues in the presence of homologous vector immunity. In other organs, BAd-GFP or HAd-GFP persisted longer in the presence of heterologous vector immunity. Conversely, rapid clearance of the vector was observed in the presence of homologous vector immunity in both HAd-GFP- and BAd-GFP-inoculated groups; although, it was not as striking for the spleen and lung of the HAd pre-immunized groups or the liver of BAd pre-immunized groups (Fig. 5c-l).

GFP mRNA expression levels in various tissues were monitored by qRT-PCR. In the tumors, GFP expression by HAd-GFP was significantly ($p < 0.05$) higher in the presence of BAd-GFP-specific immunity than in the presence of HAd-GFP-specific immunity, while GFP expression by BAd-GFP was higher in the presence of HAd-GFP-specific immunity than in the presence of BAd-GFP-specific immunity (Fig. 6a, b). In contrast, a decline in GFP expression levels was observed in the tumor tissues (Fig. 6a, b), spleen (Fig. 6d), kidneys (Fig. 6e, f), liver (Fig. 6g) and heart (Fig. 6k) of HAd-GFP or BAd-GFP inoculated mice in the presence of homologous vector immunity compared to heterologous vector immunity, although GFP expression levels in the spleen of HAd-GFP-inoculated mice were lower on Day 2 (Fig. 6c). The GFP expression levels were variable in the liver samples of BAd-GFP inoculated mice, (Fig 6h) while in the lung (Fig 6i, j) and heart (Fig. 6l) samples, transgene expression levels were relatively lower in the presence of vector immunity at several time points.

These results indicate that following i.t. inoculations both HAd-GFP and BAd-GFP persisted at a higher level in various tissues in the presence of heterologous vector immunity. The biodistribution pattern and transgene expression in tissues were adversely affected in both the presence of homologous or heterologous vector immunity suggesting the importance of anti-transgene immune response.

3.5 Histopathological evaluation following i.t. inoculations with BAd-GFP or HAd-GFP

To determine alterations in the tumor, spleen, liver, lung, kidney and heart tissues, we examined H&E stained tissue sections at various time points. At early time points (Day 0.25 to Day 4), meaningful histological differences were not observed in the tumor, spleen, lung, liver, kidney and heart samples from mice inoculated with BAd-GFP or HAd-GFP. However, a few apoptotic/necrotic hepatocytes were occasionally observed in both vector-inoculated groups. More prominent histological changes were observed in the liver at Day 8

and Day 16 post-inoculation. These changes included multifocal aggregates of macrophages and neutrophils scattered throughout the hepatic parenchyma (Fig. 7). The histopathological evaluation signifying inflammatory cell infiltration coupled with acute toxicity indicate the liver as a conspicuous site for a dynamic cellular response following i.t. inoculation with either BAd-GFP or HAd-GFP.

4. DISCUSSION

Pre-existing Ad vector immunity and immune responses to the vector backbone and transgene pose potential concerns to the repeated use of Ad vectors for cancer gene therapy. The absence of immunological cross-reactivity between HAd5 and BAd3 (Sharma et al., 2010b) coupled with the ability to efficiently transduce a variety of cell types (Bangari et al., 2005) indicate the potential of BAd3 vectors for cancer gene therapy. In this study, we modeled vector immunity in non-metastatic tumor-bearing immunocompetent FVB/n mice followed by i.t. transgene delivery in order to explore the usefulness of the BAd3 vector.

After i.t. inoculations, the biodistribution and persistence of BAd-GFP were similar to or better than those of HAd-GFP with significant proportions of both the vectors present in the tumor tissue. An apparent lack of correlation between vector genome levels and transgene expression levels (Figs. 1 and 2) could be due to variations in receptor levels, transcriptional regulations and innate immune response (Lefesvre et al., 2003; Peng et al., 2001). The results did not drastically differ from our earlier intravenous (i.v.) inoculation study (Sharma et al., 2009a) and demonstrated that vectors had spread to various organs following i.t. inoculation. Such systemic leakage of Ad could be influenced by the tumor-type and/or size of the tumor (Hiltunen et al., 2000; Wen et al., 2003; Wood et al., 1999). A mechanism for systemic virus dissemination in solid tumors has been linked to infusion-induced convection into leaky blood vessels (Wang et al., 2003; Wang et al., 2005).

The differences in the levels of chemokines and TLR expression after local (i.t.) or systemic (i.v.) (Sharma et al., 2010a) inoculations of BAd-GFP presumably reflect a complex relationship between innate immunity and the route/dose of vector administration. The differences in lymphoid cell population associated with these two routes determine the potency and quality of innate and adaptive immune responses (Zinkernagel, 2000). The resultant activation of innate immunity by Ad vectors could provide an adjuvant effect for overcoming immuno-tolerance and initiate an anti-tumor immune response (Geutskens et al., 2000).

A transient increase in the serum levels of hepatic enzymes (AST and ALT), a known feature of Ad vectors, was observed in i.t. mice. BAd-GFP did not have a significant impact on the KCs number in the liver; however, histological changes indicating mild hepatotoxicity were observed at Day 8 and Day 16. On the contrary, HAd-GFP-inoculated mice did not show fluctuations of hepatic enzymes but revealed a decline in KCs and histopathological alterations. The i.v. inoculations with HAd-GFP (Sharma et al., 2010a) also had an adverse effect on the KCs numbers and histopathological alterations indicating that both the vector and the route of inoculation play a role in liver pathology although with some differences. The underlying mechanisms for such differences possibly include the inflammatory and innate immune responses, since a HAd5 vector with a human-bovine chimeric fiber has shown reduced innate immune responses (Rogee et al., 2010). In addition, the relatively long fiber of BAd3 (Ruigrok et al., 1994) may be involved in mediating hepatic sequestration and hepatotoxicity similar to the long fiber shaft of other HAd types (Shayakhmetov et al., 2004) that exhibit hepatic sequestration and hepatotoxicity.

We observed a decline in HAd-GFP and BAd-GFP transduction and transgene expression after the repeat inoculation of these vectors in the presence of homologous vector immunity. In the liver, an unpredictable pattern of the transgene expression (Fig. 6g, h) suggest a dynamic interplay between tissue repair responses and the role of resident/migrating macrophages and neutrophils. Supporting this notion is the presence of hepatocellular necrosis/apoptotic cells, an early decline in KCs and a late increase in infiltrating cells in the liver (Figs. 4 and 7). Importantly, in the presence of heterologous vector immunity, HAd-GFP or BAd-GFP showed efficient local (tumor) and systemic tissue transduction, vector persistence and transgene expression. Since both human and non-human Ad vectors induce strong vector-specific immune responses, the success of gene therapy is contingent upon the continued delivery of a number of Ad vectors with sequential administration for a desired therapeutic effect. The persistence and transgene expression of BAd-GFP in the presence of HAd-GFP vector immunity strongly indicates the suitability of BAd3 vectors for cancer gene therapy applications where sequential vector inoculations are required.

5. CONCLUSION

The i.t. inoculations with BAd-GFP show vector persistence and transgene expression in tumors for at least two weeks in addition to vector distribution to vital organs - a pattern comparable to that of HAd-GFP. The persistence within tumor tissue and a robust transgene expression indicate that the BAd3 vector is a suitable delivery system for cancer gene therapy. Furthermore, the BAd3 and HAd5 vectors show increased transgene expression and persistence in the presence of HAd5 or BAd3-specific pre-existing immunity, respectively, suggesting that sequential administration of these vectors is a viable strategy to overcome heterologous vector immunity and to achieve prolonged transgene expression.

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HIGHLIGHTS

- We comparatively tested intratumoral inoculations of a bovine adenovirus serotype 3 (BAd3) and human adenovirus serotype 5 (HAd5) vectors in an immunocompetent mice model of non-metastatic breast cancer.
- Vector biodistribution and transgene expression patterns were evaluated in the absence or presence of pre-existing immunity.
- Higher transgene expression was observed in mammary tumors and other organs in heterologous vector immunity-simulated groups.
- The data suggests sequential administration of BAd3 and HAd5 vectors as a potential strategy to evade vector immunity and to prolong transgene expression.

Figure 2. Transgene expression analysis following HAd-GFP or BAd-GFP i.t. inoculation in the tumor tissue in the absence of pre-existing vector immunity
FVB/n mice bearing tumors were inoculated i.t. with HAd-GFP or BAd-GFP. The tumor (a), spleen (b), kidney (c), liver (d), lung (e), and heart (f) tissues were collected at indicated times post-inoculation and used for extraction of the total cellular RNA. A comparative quantification of GFP mRNA expression was performed by quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis using the total cellular RNA samples. An endogenous housekeeping gene for 18S RNA was utilized as a normalizing control for every sample. Values are reported as the average $\pm$ standard deviation of three mice at each time point with respect to transgene expression levels of HAd-GFP or BAd-GFP at Day 0.25 as 1000 arbitrary units. * $P < 0.05$ HAd-GFP vs. BAd-GFP at indicated time points.

Figure 3. Immunohistochemical analysis of GFP expression in the tumor and liver of mice inoculated with HAd-GFP or BAd-GFP

Immunohistochemical analysis for GFP protein expression was performed on formalin fixed and paraffin-embedded tumor and liver tissue sections of mice at Day 0.5 post-inoculation with HAd-GFP or BAd-GFP as described in Materials and Methods. Positive staining is indicated by a reddish brown coloration, and nuclei are stained blue with hematoxylin. The experiment was performed on three mice in the group with similar results.

Figure 4. Evaluation of innate immunity and toxicity due to i.t. inoculation of HAd-GFP or BAd-GFP

To determine the activation of innate immune response in the tumor or spleen following i.t. inoculation, total cellular RNA was isolated from the tumor and spleen tissue of mock (PBS), HAd-GFP, or BAd-GFP inoculated mice at Day 0.25. A quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed using 200ng of total RNA. Gene expressions for various cytokines, chemokines (a & c), TLRs and the adapter molecules (b & d) were monitored. The data was normalized to the 18S endogenous control, and values are reported as mean percent $\pm$ standard deviation from three mice with respect to the PBS-inoculated control mice (as 100%). Blood samples were collected after i.t. inoculation of mice with HAd-GFP or BAd-GFP and analyzed for alanine aminotransferase (ALT) (e) and aspartate aminotransferase (AST) (f) levels. An immunohistochemical analysis for F4/80 positive KCs was performed on formalin-fixed and paraffin-embedded liver tissues. Anti-F4/80 stained cells are indicated as the cells showing a reddish brown color, and the nuclei are stained blue with hematoxylin (g). Five random fields were selected for each section, and the number of KCs was estimated by counting cells in a field at 600 $\times$ magnification. The values are reported as mean $\pm$ standard deviation for three mice per group.

Figure 5. Comparative analysis of biodistribution and persistence of HAd-GFP and BAd-GFP in the presence of vector immunity

Mice were first inoculated i.m. with HAd-GFP or BAd-GFP to develop vector immunity followed by a second inoculation (i.t.) with HAd-GFP or BAd-GFP. Genomic DNA was isolated from the tumor, spleen, kidney, liver, lung and heart tissues after the second vector inoculation at the indicated time points. HAd-GFP or BAd-GFP copy numbers in the tumor (a & b), spleen (c & d), kidney (e & f), liver (g & h), lung (i & j) and heart (k & l) tissues were determined by quantitative real-time PCR (qPCR) using vector-specific primers and a probe for the E4 region. Serial dilutions of purified vector DNA were used as standards in every experiment, and the quantification of the vector genome was extrapolated with respect to the standard curve. The amount of residual vector genome from the first inoculation was determined in parallel at each time point in separate groups of mice inoculated a second time

with PBS. The residual genome from the first inoculation was subtracted from the repeat vector inoculation groups to determine the final absolute copy number. Values are derived from the average $\pm$ standard deviation of three mice at indicated time points. * $P < 0.05$ HAd-GFP vs. BAd-GFP at indicated time points.

Figure 6. Transgene expression analysis following HAd-GFP or BAd-GFP i.t. inoculation in the tumor tissue in the presence of vector immunity

Mice were first inoculated i.m. with HAd-GFP or BAd-GFP to develop vector immunity followed by an i.t. inoculation with HAd-GFP or BAd-GFP. Total cellular RNA was isolated from the tumor, spleen, kidney, liver, lung and heart tissues after the second vector inoculation at the indicated time points. A comparative quantification of GFP mRNA expression was performed by real-time reverse transcriptase PCR (q-RT-PCR) using the total RNA of the tumor (a & b), spleen (c & d), liver (e & f), kidney (g & h), lung (i & j) and heart (k & l) tissues. An endogenous housekeeping gene for 18S RNA was utilized as a normalizing control for every sample. Relative RNA expression was quantified and calibrated with respect to the residual gene expression (from the first inoculation) present in the Day 0.25 PBS-inoculated control samples of the repeat inoculation groups. Values are

reported as the average $\pm$ standard deviation of three mice at each time point. * $P < 0.05$ HAd-GFP vs. BAd-GFP at indicated time points.

Figure 7. Histopathological examination following i.t. inoculation of HAd-GFP and BAd-GFP vectors

The hematoxylin and eosin stained sections from the tumor, spleen, kidneys, liver, lungs and heart tissues at every time point in the single inoculation (i.t) or repeat (i.m followed by i.t.) vector inoculations were examined by a board-certified pathologist (DSB). The most prominent changes were observed at Day 8 post vector inoculation in the liver parenchyma. The images are shown with 200× magnification; the top label indicates the first inoculation and side label indicates the second inoculation. The cellular infiltration in hepatic parenchyma with a magnified image is shown as an inset.