

ADENOVIRUS VECTOR GENOME EVALUATION AFTER INTRAVITREAL ADMINISTRATION

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Introduction

Preclinical data have indicated that expression of PEDF from an adenoviral vector has promise as an experimental treatment for ocular neovascular disease. Expression of many genes under the direction of the CMV promoter in an adenoviral vector has been reported to be transient in nature lasting approximately 2 weeks after administration into the vitreous of the eye. The loss of expression could be due to the clearance of vector genomes from the eye or the shut off of expression from the vector genomes. To address these possible mechanisms we measured the presence of vector genomes after intravitreal delivery.

These studies are focused on the development of assays and techniques to quantitatively detect adenovector genomes and on the evaluation of the vector genome over time post intravitreal administration in the mouse eye. If genomes are present in the eye they represent a potential source for expression of a therapeutic molecule. We chose to evaluate an E1, E3, E4 deleted adenovector that expresses luciferase from the CMV promoter (AdL.11D). The difference between AdL.11D and our lead molecule AdPEDF.11D is the gene that is expressed. The adenovirus vector base and expression cassette are identical.

Materials and Methods

ANIMALS. All animal protocols and handling were performed using GenVec approved protocols. In order to ensure integrity of the samples and to limit cross contamination issues great care was taken in the administration and tissue preparation for these experiments. These techniques are necessary for the sensitive nature of the assays being conducted.

IN-VIVO VECTOR ADMINISTRATION. Various doses of AdL.11D were administered in a 2µl volume into the vitreal cavity of the right eye of C57BL/6 female mice that were 6-8 weeks old. Animals were sacrificed at the designated time post administration and tissue harvested.

DNA EXTRACTION. Tissues were ground on dry ice in a mortar and pestle. The mortar and pestle were pre-cooled with liquid nitrogen before being placed on dry ice. Between each sample the operator changed gloves along with mortar and pestle. The ground samples were placed back at -80°C before DNA extraction occurred. DNA was extracted from tissue following a proteinase K protocol which yields a high quality and high levels of DNA. An alternative protocol (Qiagen DNeasy Tissue purification kit) was used in some experiments. The Qiagen DNeasy protocol for Animal tissue is a simple procedure that gives consistent results. The DNeasy Tissue Kit uses advanced silica-gel-membrane technology for rapid and efficient purification of total cellular DNA without organic extraction or ethanol precipitation. The DNA isolated by the proteinase K method after RNase treatment gave a 1 log higher background than the DNA purified by the Qiagen DNeasy Tissue purification kit (data not shown).

SOUTHERN PROTOCOL.

Probe preparation. The pIX region of Ad5 was subjected to PCR (94°C 5 min, 30x [94°C 30 sec, 55°C 30 sec, 72°C 30 sec] in a 100 µl volume using primers A2a4022B (CGGGATCCTTAAACCGCATTTGGGAGGGAGG) and A2s3595B (CGGGATCCGGCCATGAGCGCCAACTCGTTTG) using Taq polymerase (BMB) following manufacturer's protocol. The DNA was resolved on a 1% agarose gel in 0.5% TBE (Amersco) and purified through a QIAquick Gel Extraction kit column (Qiagen #28074) and DNA eluted following manufacturer's protocol. The purified DNA was radiolabelled with ³²P using a Rediprime II kit (RPN 1633) from Amersham following manufacturer's protocol.

Gel Preparation. 10 µg of total DNA purified by the proteinase protocol was cut with KpnI (NEB) to completion before being resolved on a 1% agarose gel in 0.5% TBE buffer (Amersco).

Transfer and Probing. The Southern blot protocol published by Bio-Rad (Lit234 rev B) sections 2.3 (DNA Capillary Transfer), and 4.1 (Standard Protocol [Prehybridization, Hybridization, and Washes]) was followed with modifications. The solid support was Nytran N nylon membrane from Schleicher and Schuell (10416089). The prehybridization buffer is 0.25 M sodium phosphate (pH 7.2), and 7% SDS. The 1st wash buffer is 20mM sodium phosphate (pH 7.2) and 5% SDS. The 2nd wash buffer is 20mM sodium phosphate (pH 7.2) and 1% SDS.

Image acquisition. To visualize the data Kodak XAR-5 film was exposed to the southern blot at -80°C with an intensifying screen before being developed. Quantitation of the Southern blot was carried out with a Packard Instant Imager following manufacturer's recommendations.

QUANTITATIVE PCR PROTOCOL.

Probe and primer design. qPCR primers and a probe were designed with the aid of the primer express software from ABI. The pIX region specific amplicon primer and probe sequences designed to detect the left region of the vector genome are as follows. Forward primer: 5'-CGCGGATTGTGACTGACT-3', Reverse primer: 5'-GCCAAAGAGCCGTCACCTT-3', Fluorogenic Probe 5'-FAM-AGCAGTGCAGCTTCCCGTTTCATCC-TAMRA-3'. The 11D specific amplicon primer and probe sequences were designed to detect the right end of the vector genome are as follows. Forward primer: 5'-TTGGGAAGACAATAGCAGGC-3', Reverse primer: 5'-GGGTTTCTACAGGACGGACC-3', Fluorogenic Probe: 5'-FAM-CTCACTATACGGATCTGCCATCATGGCC-TMRA-3'.

Quantitative Polymerase Chain Reaction. Each sample was assayed in duplicate. Experimental, naïve tissue and a no template control were included in each assay set. The reactions were thermal cycled, recorded, and analyzed using the ABI PRISM 7700 Sequence Detection System. A standard curve was generated using eleven serial dilutions of the pAdE1(L)E3(10)E4(TIS1) plasmid DNA in 1 µg of genomic liver DNA. The dilution series represented the range from 1000000 copies to 100 copies of the plasmid. In addition, a negative control reaction containing no template was included. The quantitative PCR reactions were assembled based upon the Taqman PCR Core Reagent kit and Taq Man Universal PCR Master Mix kit protocol (PE Applied Biosystems). The reactions were run in duplicate in adjacent wells. Final concentration of each primer was 200 nM each and 100 nM for the probe. The reactions were thermal cycled using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Data were collected by the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) to generate a standard curve with correlation coefficient and slope determined. The threshold cycles (Ct) of the no template control reactions were at 40 cycles.

Analysis. Quantitative PCR is a real-time quantitation detection system involving the 5' to 3' nuclease activity of Taq DNA polymerase. The real-time aspect is accomplished by setting the threshold cycle at a point in the exponential phase of amplification. The precision of the results is determined by the standard curve slope and correlation coefficient. The samples are compared to the standard curve. The samples are quantified according to their standard deviation and mean in relation to the standard curve.

For the tissue samples, 52 ng DNA of total DNA was used per reaction. The standard curve was generated using dilutions of 100 to 1000000 copies of pAdE1(L)E3(10)E4(TIS1) in 1 µg of genomic liver DNA.

Analysis of Adenovector Genomes by Southern

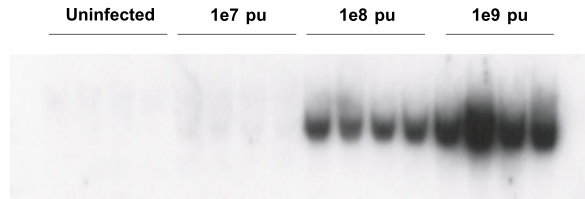


Figure 1. The relative level of vector genome delivered to the eye was determined by Southern blot analysis. Southern blot analysis provides a method of determining relative genome levels while simultaneously confirming genome integrity. Doses of 1e7, 1e8 and 1e9 particle units (pu) of AdL.11D were administered to the vitreous of the eye and on day 1 post vector administration eyes were harvested, DNA isolated and Southern blot analysis performed. The strong single band is the same size as the positive control (data not shown).

Quantitative Analysis of Southern Results

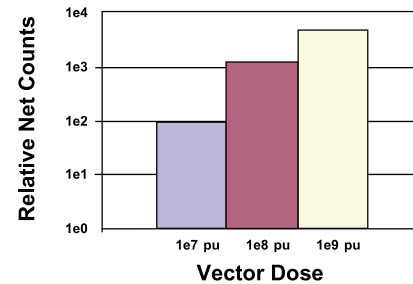


Figure 2. Phospho-imaging quantitation of Southern results from two experiments is summarized. The 1e7 pu dose was found to be above background in these analyses although not visibly detected by the autoradiographic techniques used to generate figure 1. The limit of detection using this technique appears to be near 1e7 pu of delivered vector. There is a ~10 fold increase in genomes detected between 1e7 and 1e8 pu administered with an additional ~4-5 fold increase in genomes detected between the 1e8 and the 1e9 vector doses.

Quantitative Analysis of Adenovector Genomes by qPCR (left end detection)

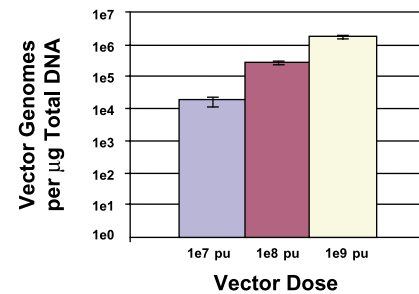


Figure 3. A quantitative PCR (qPCR) approach to adenovector genome detection was compared to the Southern approach. A series of experiments were undertaken to determine the utility of qPCR techniques for adenovector genome quantitation in the eye. 1e7 to 1e9 pu of AdL.11D vector was administered to the vitreous of the eye and at 1 day post administration vector genome levels were quantitated using qPCR assay specific to the left end of the vector. The 1e7, 1e8 and 1e9 doses yielded 1.8e4, 2.6e5 and 1.7e6 genomes per µg of total DNA respectively. The difference in level of genomes detected between 1e7, 1e8 and 1e9 doses was as predicted by the 10 fold increase in vector administered. These results are in good agreement with that found with the Southern blot approach and suggest that this qPCR approach reliably detects the amount of vector genome present.

Quantitative Analysis of Adenovector Genomes by qPCR (right end detection)

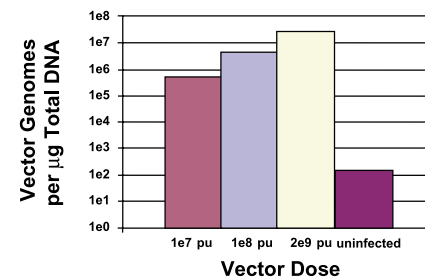


Figure 4. A quantitative PCR (qPCR) approach specific to the right end of the vector was analyzed and compared to the Southern approach. A series of experiments were undertaken to determine the utility of qPCR techniques for adenovector genome quantitation in the eye. 1e7 to 2e9 pu of vector was administered to the vitreous of the eye and at 1 day post administration vector genome levels were quantitated using qPCR primers and probes specific to the right end of the vector. The difference in level of genomes detected between doses was as predicted and compared to that seen previously with the left end specific qPCR approach. These data support the use of our qPCR methods to quantitate vector genomes in the eye and suggest that these approaches will be useful to quantitate levels of adenovector in the eye and is a very sensitive method.

Adenovector Genome Clearance and Persistence

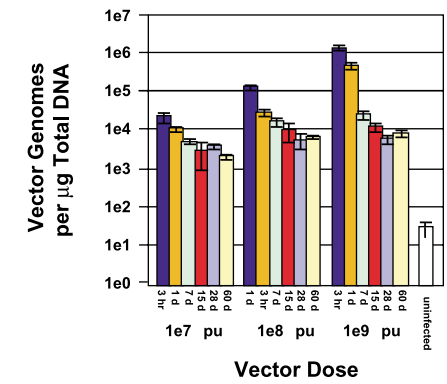


Figure 5. The amount of adenovector genome present in the eye was followed for 2 months after administration. At 3 hours, 1 day, 7 days, 15 days, 28 days, and 60 days after administration to the vitreous the amount of vector genome was determined by qPCR. The amount of vector genome detected was proportional to the dose administered at 3 hours post administration. A dose dependent loss of genomes is seen between 3 hours and 15 days post administration. Interestingly the amount of vector genome stabilizes after 15 days post administration and is maintained at the same level for 60 days for each of the 3 doses (1e7, 1e8, and 1e9 pu) examined.

Treatment	Ocular Histopathology	Systemic Histopathology	Clinical Chemistry	Hematology
naive	No abnormalities reported	No abnormalities reported	No abnormalities reported	No abnormalities reported
1e6 pu of AdPEDF.11D	No abnormalities reported	No abnormalities reported	No abnormalities reported	No abnormalities reported
1e8 pu of AdPEDF.11D	No abnormalities reported	No abnormalities reported	No abnormalities reported	No abnormalities reported

Figure 6. Histopathology, clinical pathology, hematology, cage side observations, and morbidity following administration showed no abnormal responses following administration of either 1e6 or 1e8 pu at 4, 29, 51 and 91 days post delivery to the eye. Experiments evaluating the safety of adenovector in mice following administration to the vitreous showed that adenovectors are well tolerated.

Conclusions

- Intravitreal injection of 1e6 and 1e8 pu of AdPEDF.11D resulted in no effects on any parameters of clinical pathology, hematology, clinical chemistry, or histopathology observed at any time point.
- Vector genome in the eye at 3-hour and 1-day post administration correlated directly with the amount of vector particles administered and the amount of vector genome remained remarkably constant from 15 to 60 days post administration.
- These findings suggest that adenovectors may provide a means to deliver proteins to the eye requiring longer-term expression for the treatment of ocular disease. Further studies are underway.

