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Complete replication-competent adenovirus 11p vectors with E1 or E3 insertions show improved heat stability

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ABSTRACT

Conventional adenovirus vectors harboring E1 or E3 deletions followed by the insertion of an exogenous gene show considerably reduced virion stability. Here, we report strategies to generate complete replication-competent Ad11p(RCAd11p) vectors that overcome the above disadvantage. A GFP cassette was successfully introduced either upstream of E1A or in the E3A region. The resulting vectors showed high expression levels of the hexon and E1genes and also strongly induced the cytopathic effect in targeted cells. When harboring oversized genomes, the RCAd11pE1 and RCAd11pE3 vectors showed significantly improved heat stability in comparison to Ad11pwt;of the three, RCAd11pE3 was the most tolerant to heat treatment. Electron microscopy showed that RCAd11pE3, RCAd11pE1, Ad11pwt, and Ad11pE1 Delmanifested dominant, moderate, minimum, or no full virus particles after heat treatment at 47 °C for 5 h. Our results demonstrated that both genome size and the insertion site in the viral genome affect virion stability.

1. Introduction

Oncolytic or replication-competent adenovirus (Ad) vectors are considered promising virotherapy vehicles of high utility because they can be easily constructed, are produced at high titers, and can efficiently transduce various types of cells. Most commonly, the Ad vectors that are used for clinical purposes are based on adenovirus serotype 5 (Ad5), which belongs to subgroup C. In recent years, however, it has become apparent that Ad5 vectors have some drawbacks, such as the high seroprevalence of anti-Ad5 antibodies in adult humans and the low transduction efficiency of Ad5 vectors in cells lacking the primary receptor for Ad5, the coxsackievirus and adenovirus receptor (CAR), which is the case for numerous types of metastatic solid tumors and leukemia cells. This critical roadblock could be overcome by using an alternative serotype of the virus to bypass the presence of pre-existing antibodies that have been elicited by previous natural Ad infections. The replication competent Ad11 vector could be used to overcome the disadvantages associated with Ad5 vectors, as species B Ad11 also uses DSG-2 and CD46 as receptors (Wang et al., 2011), which are ubiquitously expressed on metastatic tumor cells, and Ad11 shows a low seroprevalence in humans. Indeed, only approximately 10–31% of the population is seropositive for Ad11, whereas up to 90% of the population is seropositive for Ad5. Notably, there is no serum cross reactivity between Ad11 and Ad5 (Holterman et al., 2004; Stone et al., 2005).

A number of species B Ads, such as Ad3, Ad7, Ad11 and Ad35, have been developed as gene therapy or vaccine vectors. Such vectors have primarily been designed according to conventional methodology: they incorporate an E1 deletion followed by substitution with an expression cassette. Such vectors display characteristics common to Ad, showing low production and heat lability, and they satisfy the requirements needed to produce virus in packaging cell lines. Replication-competent Ad11 vectors have also been reported by Wong et al. (2012). To create these vectors, the promoter-enhancer and promoter sequence from 195 nt to 358 nt of Ad5 were replaced with a sequence fragment from 249 nt to 392 nt of Ad11p, resulting in Ad11p-Ad5-EP vectors that could replicate in a greater variety of cancer cell lines than the parent viruses.

Ad E1A, a multifunctional protein expressed early after infection, interferes with numerous important regulatory processes by interacting with host cell proteins or directly transcriptionally activating target genes. Although E1A was initially identified as an

Abbreviations: Ad, Adenovirus; Ad11pwt, Wild type Ad11 prototype; GFP, Green fluorescence Protein; RCAd11pE1GFP, E1GFP, Replication-competent Ad11p with E1 GFP insertion; RCAd11pE3GFP, E3GFP, Replication-competent Ad 11p with E3 GFP insertion; Ad11pE1DelGFP, E1DelGFP, Replication incompetent Ad11p with E1 deletion and GFP insertion

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adenoviral component that can cause the malignant conversion of rodent cells, this protein has also been shown to possess remarkable tumor suppressive effects on various types of human cancer cells (Frisch, 1991; Frisch and Mymryk, 2002). For instance, E1A down-regulated HER2/neu expression in addition to participating in other antitumor mechanisms unrelated to Her-2 (Chang et al., 1997). E1A also negatively regulates cellular proteins that are important for gene transcription, such as p300/CBP, TATA-binding protein, TBP-associated factors, NF-kB, ATF-4 and c-Jun (Brockmann et al., 1995; Chen and Hung, 1997; Liang and Hai, 1997). The E1A protein has also been shown to induce sensitization to anticancer drug-induced apoptosis, as well as to reduce tumor metastasis and promote apoptosis under conditions of serum deprivation caused by the application of tumor necrosis factor a (TNF-a), irradiation or anticancer agents such as Taxol, etoposide, and Adriamycin. For these reasons, E1A-based viral gene therapies have been extensively exploited in both pre-clinical and clinical settings. Thus, there is a need to keep the E1 gene intact, and recent studies have focused on creating Ad vectors that include a therapeutic gene insertion in the E3 region as opposed to in the E1 region. The E3 region of the Ad genome has been previously shown as unnecessary for virus replication and can therefore be deleted to introduce a large expression cassette for therapeutic purposes. However, this concept has been challenged by recent reports indicating that the re-introduction of full-length E3 back into the virus genome increases its therapeutic efficacy (Danielsson et al., 2008). Thus, the deletion of E3 may affect viral function.

From the perspective of studying the oncolytic capacities of Ad vectors, the receptor-restricted expression of Ad is unfortunate. Wild-type Ad can induce oncolysis in some cells, but it is not possible to directly quantify the receptor-restricted transduction of the virus. In contrast, replication-defective Ad vectors with quantifiable marker genes in the E1 region (i.e., GFP or luciferase) can be monitored for viral entry and gene expression in the nucleus, but their cytolytic properties are suboptimal due to the lack of expression of the E1A transactivating gene. In addition, the replication capacities of such defective vectors are considerably lower than that of wild-type virus. Thus, these defective vectors cannot be reliably used as a control to study Ad cytolysis.

Many studies have recently focused on the development of recombinant Ad vectors harboring modifications of the E1 promoter (Gaynor et al., 1982; Grable and Hearing, 1990; Osborne et al., 1982); however, no report has yet addressed the possibility of inserting a reporter gene into the full-length E1 region without disrupting either the function of the packaging signal or the transactivation activity of the product of the E1A open reading frame (ORF). For the E3 Ad vector, commercial deletion of the E319K ORF reduced cytotoxicity in MHC class I presenting cells, a finding that might not be suitable for oncolytic purposes. To overcome these limitations, in the current work, we present novel strategies for the insertion of expression cassettes without any deletion of the E1 region or with limited deletion of the E3 region in the Ad 11p genome; we designated these constructs replication-competent adenovirus E1GFP (RCAd11pE1GFP) and replication-competent adenovirus E3GFP (RCAd11pE3GFP), respectively. These replicating Ad11p viruses possess wild-type viral replication capacity, high GFP expression, and enhanced virion heat stability.

2. Results

2.1. Characteristics of RCAd11pE1GFP, RCAd11pE3GFP, and Ad11pe1DelGFP vectors

The strategies used to construct recombinant replication-competent adenovirus 11p e1 GFP (RCAd11pE1GFP or E1GFP), and replication-competent adenovirus 11p e3 GFP (RCAd11pE3GFP or E3GFP) vectors are illustrated in Fig. 1A–D. The construction of replication-incompetent adenovirus 11p E1Del GFP (Ad11pE1DelGFP or E1DelGFP) vector is described in materials and methods. The genome sizes were 36,523 bp for RCAd11pE1GFP, an increase of 4.97% over the unmodified Ad11p wild type genome; 35,403 bp for RCAd11pE3GFP, an increase of 1.75% over the Ad11p genome; and 33,624 bp for Ad11pE1Del GFP, a decrease of 3.36%, compared to the Ad11p genome. Additional biological characteristics of the Ad11p vectors, such as insertion sites, GC contents, deletions, modifications, and packaging cell lines, are summarized in Table 1.

2.2. The E1 insertion did not disrupt transcription of the E1A and E1B genes

A549 cells infected with Ad11pwt, RCAd11pe1 and RCAd11pE3 viruses showed successful transcription of the E1A gene. Using RT-PCR, two E1A splice forms were identified at 7 h p.i. at the indicated 268R and 231R fragments. By increasing the incubation time to 24 or 48 h, an additional E1A fragment of 58R could be visualized on an agarose gel. Similarly, the ability of the RCAd11pGFP and Ad11pwt viruses to produce E1B 55k and E1B pIX mRNA in virus-infected cells was also detected by RT-PCR using two pairs of specific primers. As shown in Fig. 2A, the 55 K and pIX ORFs were both detectable at 24 h p.i., and their transcript levels increased by 48 h p.i. A slight reduction in the E1B ORF transcript levels for RCAd11pe1GFP was observed in the RT-PCR assay. However, all detected mRNAs induced by Ad11pwt or RCAd11pGFPs were highly similar and comparable (Fig. 2A).

2.3. qPCR to detect E1A and hexon gene expression in A549 cells

To ensure that the insertion upstream of the E1A gene in the RCAd11pE1 vector does not negatively affect virus replication, we analyzed E1A mRNA levels by quantitative reverse-transcription PCR using Ad11pE1A-specific primers. Ad11pwt and RCAd11pE3 vectors possess an identical E1A sequence, and the qPCR results showed similar levels of E1A gene expression at time points of 2, 8, 24 and 48 h. In contrast, the RCAd11pE1 vector, which carries an additional GFP sequence upstream of the E1A region, showed relatively higher E1A expression at 2 h p.i. Interestingly, the cells infected with the RCAd11pE1 vector also showed higher levels of E1A gene expression than the cells infected with the Ad11pwt or RCAd11pE3 vectors at 48 h and 72 h p.i. Not unexpectedly, E1A gene expression ranged from log 4 to log 5, and hexon gene expression ranged from log 5 to log 7 at 24 h and 72 h p.i. In general, the Ad11pwt, E1GFP, and E3GFP viruses led to comparable levels of E1 and hexon gene expression (Fig. 2B and C).

2.4. Kinetics of GFP expression following infection with RCAd11pE1 or RCAd11pE3 viruses in A549 cells

Electron microscopy images of virions from the RCAd11pE1 vector showed intact capsids with classical species B Ad morphology. Virions with full genomes and short fibers as well as viruses lacking DNA were observed, as shown in Fig. 3A. The virus products from each vector were compared with that of wild-type Ad11p in A549 cells, and the multiplicities of infection for each viral preparation were determined by $TCID_{50}$ values for both A549 and HEK293 cells. A total of 5.4×10^{13} vp or 15 mg virion/batch was obtained from each preparation. The ratio of infectious particles to physical particles was further studied using a $TCID_{50}$ assay; one infectious particle among 72 viruses or less was detected for the two Ad11pGFP vectors as well as the Ad11pwt virus. This result was in agreement with our previous data (Mei et al., 1998). Thus, the inclusion of a GFP insert at the front of the E1 region or