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Establishment and validation of new complementing cells for production of E1-deleted adenovirus vectors in serum-free suspension culture

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A B S T R A C T

E1-deleted adenovirus vectors (AdV) are important gene transfer vehicles for gene therapy and vaccination. Amplification of AdV must take place in cells that express the adenovirus E1A and E1B genes. Sequence homology between AdV and the E1 genes integrated within the complementing cells should be minimal to reduce the odds of generating replication-competent adenovirus (RCA). The present study describes the establishment of AdV complementing cells constructed by stable transfection of the minimal E1A and E1B genes into human lung carcinoma (A549). Because some transgene products can be cytotoxic, the cells were engineered to stably express the repressor of the cumate-switch (CymR) to silence transgene transcription during vector growth. For regulatory compliance and to facilitate the scale-up, the resulting complementing cells (SF-BMAdR) were adapted to serum-free suspension culture. The best clone of SF-BMAdR produced AdV carrying an innocuous transgene to the same level as 293 cells, but titers were better for AdV carrying transgene for a cytotoxic product. Elevated titers were maintained for at least two months in suspension culture in the absence of selective agent and the cells did not produce RCA. Because of their advantageous properties, SF-BMAdR cells should become an important tool for developing large-scale production processes of AdV for research and clinical applications.

1. Introduction

Several advantageous features are responsible for making vectors derived from adenovirus (adenovirus vectors, AdV) an excellent vehicle for gene transfer and vaccination (reviewed in (Khare et al., 2011). For example, AdV can be grown and purified to very high titers (above 1.0×10^{12} virus particles per ml); the virion, being relatively stable, can sustain long-term storage as well as several cycles of freeze/thawing without losing infectivity. They can efficiently transduce a battery of cell types and tissues (either actively dividing or at rest). Importantly, the fact that the great majority of vector genomes remain as non-integrated extra chromosomal nuclear elements after transduction greatly reduces the odds of insertional mutagenesis due to random integration. E1-deleted AdV, also known as first generation AdV, carry viral genes that are expressed at low level after transduction. Leaky viral gene expression induces an immune response that can be toxic and that prevents stable long-term transgene expression. For this reason, first generation AdV and those that were subsequently engineered to re-express the E1 region, are actively developed as vaccine for cancer and infectious diseases or as oncolytic agents, where high-level but transient gene expression is beneficial (Rollier et al., 2011; de Gruijl and van de Ven, 2012; Crystal, 2014).

Abbreviations: AdV, adenovirus vector; Ad5, adenovirus type 5; CymR, cumate-switch repressor; CD::UPTR, cytosine deaminase::uracil phosphoribosyl transferase gene; CR5, cumate inducible promoter; FBS, fetal bovine serum; GFP, green fluorescent protein; HDEP, helper dependent E1-positive particles; IRES, internal ribosome entry site; ITR, invert terminal repeats; LC-SFM, low calcium hybridoma serum-free culture medium; MOI, multiplicity of infection; PRO293, PRO293S CDM culture medium; PP, physical particle; RCA, replication-competent adenovirus; SFM4-T, SFM4Transfx-293 culture medium; TU, transducing units.

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In a typical E1-deleted AdV, the gene to be expressed (transgene) is carried by the vector in place of the essential E1 region (E1A and E1B genes). Amplification of this type of AdV must take place in complementing cells that provide the E1 functions in *trans* such as for the familiar 293 cells, that were established by stable transfection of human embryonic kidney cells with a segment of the adenovirus genome corresponding to the left-hand of the vector (Graham et al., 1977; Louis et al., 1997). AdV have flanking homology sequences with the adenovirus fragment integrated into 293 cells. Acquisition of the E1 region through homologous recombination results in the emergence of replication-competent adenovirus (RCA) (Lochmuller et al., 1994; Hehir et al., 1996; Zhu et al., 1999). To prevent the formation of RCA during vector growth, a few complementing cell lines have been generated with reduced homology between the integrated adenovirus sequence and the vector (Fallaux et al., 1998; Gao et al., 2000; Kim et al., 2001; Farson et al., 2006; Xu et al., 2006; Howe et al., 2006; Kovesdi and Hedley, 2010). Two of these cell lines were also adapted to grow in suspension culture and in serum-free medium for ease of scale-up and for regulatory compliance (Xie et al., 2002; Farson et al., 2006).

Products of transgenes carried by AdV can be cytotoxic to cells, and their synthesis during AdV growth can dramatically reduce the yield of vectors (Matthews et al., 1999; Kagawa et al., 2000; Bruder et al., 2000; Gu et al., 2002; Gall et al., 2007; Zhao et al., 2009). A solution for this problem is to silence transcription of the transgene by engineering a complementing cell line synthetizing a repressor specific for the transgene promoter (Matthews et al., 1999; Gall et al., 2007; Zhang et al., 2008; Zhao et al., 2009). Our research team has developed an inducible gene transcription system that is regulated by the addition of a small organic compound known as cumate (Mullick et al., 2006). In one configuration of the cumate-switch, a repressor (CymR) specifically binds to a modified version of the CMV early enhancer/promoter (CMV5CuO) and prevents transcription. Importantly, none of the AdV complementing cell lines that were constructed to prevent the formation of RCA was designed to improve the production of AdV carrying cytotoxic transgene products. The present study describes the construction of new producer cells (SF-BMAdR cells) having such properties. These cells were made by stable integration of EA1 and E1B genes of adenovirus and CymR gene into human lung carcinoma cells (A549). To facilitate scale-up of AdV and for regulatory compliance, the cells were adapted to suspension culture in serum-free media commercially available. The best clone of SF-BMAdR cells produce AdV carrying an innocuous transgene (the green fluorescence protein, GFP) to the same level as 293 cells. Notably, in the case of an AdV carrying a cytotoxic transgene product (cytosine deaminase::uracil phosphoribosyl transferase, CD::UPTR), the titers produced by SF-BMAdR cells were better than 293 cells. In conclusion, SF-BMAdR cells possess advantageous characteristics that are essential for developing an efficient large-scale production process for manufacturing safe AdV for research and clinical applications.

2. Materials and methods

2.1. Cells and culture conditions

E1-transformed human embryonic kidney 293 cells (HEK293, Graham et al., 1977), A549 and HeLa S3 cells were purchased from ATCC (Manassas, VA). These cells, 293cTA cells (Mullick et al., 2006) and BMAdE1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT) and 2 mM glutamine (Gibco, Life Technologies). 293SF cells (clone 293SF-3F6, Cote et al., 1998), SF-BMAdE1 cells and SF-BMAdR cells were grown in custom made low calcium hybridoma serum-free medium (LC-SFM, Gibco, Life Technologies) supplemented with 0.1% Bovine Serum Albumin (Sigma–Aldrich, St-Louis, MO), 1% chemically defined lipids (Gibco, Life Technologies) and 2 mM glutamine, or in SFM4Transfx-293 medium (SFM4-T, Hyclone) supplemented with 6 mM glutamine. SF-BMAdR cells were also grown in PRO293S CDM (PRO293, Lonza, Walkersville, MD) supplemented with 6 mM glutamine. Adherent cells were grown in treated 6-well plates or 10-cm plates (Nunc, Penfield, NY). Suspension cultures were performed in 6-well plates (Sarstedt), or in 125 ml shake flasks (Corning, Acton, MA) for long-term cultivation (stability study) and for the growth curve analysis. Amplification to make cell banks was done in 125 ml shake flasks. Agitation was done at 100–120 rpm using orbital shaker (Bellco, Vineland, NJ or GL-300 Analytiqs, Montréal, Québec). Cell banks of frozen vials were made in culture medium supplemented with 7.5% DMSO (Sigma–Aldrich) and kept frozen in liquid nitrogen. All cells were cultivated at 37 °C under 5% CO_2. Cell doubling time was calculated using the following formula: doubling time = $T(x)\ln 2/\ln(Xe/Xb)$, where T is the incubation time, Xb is the cell number at the beginning and Xe is the cell number at the end of incubation time.

2.2. Plasmids

Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA) or Boehringer-Mannheim Inc-Roche (Laval, QC, Canada) and used according to the supplier's recommendations. Plasmids were constructed using standard methods of molecular biology. Plasmid DNA was either purified by CsCl-ethidium bromide density gradient centrifugation (plasmid pHβE1AE1B) or by affinity chromatography (Plasmid Mini and Maxi kits, Qiagen, Mississauga, ON, Canada). The plasmid pHβE1AE1B was constructed by subcloning the 3.0 kb genomic DNA of adenovirus type 5 (Ad5) E1 region (nucleotide number: 532-3525) as a *SalI-BamHI* fragment into the *SalI-BamHI* cloning sites of pHβApr-1-neo expression vector (Gunning et al., 1987). These restriction sites were introduced by site-directed mutagenesis in the plasmid pXC38 which contains the Ad5 E1 region from 1-5788 (*XhoI*) subcloned in pBR322 (a generous gift of Dr. P. Branton, McGill University, Montreal, QC). The site-directed mutagenesis was performed using the Transformer™ site-directed mutagenesis kit (Clontech Laboratories Inc, Mountain View, CA). Plasmid pcDNA3-55 K (a gift from Dr. P. Branton, McGill, University) carries the E1B-55 kDa gene of adenovirus regulated by the CMV promoter. Plasmid pMPG-CMV5-CymR-opt encodes the repressor of the cumate switch (CymR) (Mullick et al., 2006) regulated by the CMV5 promoter (Massie et al., 1998), and the resistance for hygromycin. It was constructed by replacing the insert of plasmid pMPGB43k (Gervais et al., 1998) with the CMV5 promoter and the codon optimized sequence of CymR ordered from GeneArt (Regensburg, Germany).

2.3. Adenoviral vectors

Unless stated otherwise, AdV were amplified on 293 cells and stocks of vectors were purified by two consecutive centrifugations on CsCl gradients using standard procedures (Acsadi et al., 1994). The purified AdV were resuspended in 20 mM Tris–HCl (pH 8), 2.5% glycerol, 25 mM NaCl and kept frozen at −80 °C. The infectious titer of the AdV (transducing units [TU]/ml) was determined by measuring the percentage of GFP or DsRed positive cells after infection of 293 and 293cTA cells by flow cytometry using an LSR II (BD Biosciences, Mississauga, Ont. Canada) or a FC 500 MPL (Beckman-Coulter, Mississauga, ON, Canada) flow cytometer, or by plaque assays (Acsadi et al., 1994; Cote et al., 1997). Isolation of adenoviral