

Adenoviral vector systems for gene therapy

Review Article

Min Wang,¹ Xinyu Zheng,¹ Xiao-Mei Rao,¹ Hongying Hao,¹ Yanbin Dong,² Kelly M. McMasters,^{1,2} and H. Sam Zhou^{1,3,4,*}

¹James Graham Brown Cancer Center,

²Department of Surgery,

³Department of Medicine,

⁴Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40202

*Correspondence: H. Sam Zhou, James Graham Brown Cancer Center, Baxter II/Room 321, Louisville, KY 40202, USA; Phone: (502) 852-5745; Fax: (502) 852-2356; Email: hszhou01@louisville.edu

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Summary

Adenoviral (Ad) vectors are widely used in gene therapies, recombinant viral vaccines, and basic science studies. The vectors can deliver therapeutic genes into cells to recover the lost function of some genes, to enhance the ability of host immune systems, or to increase the sensitivity of cancer cells to chemotherapeutic drugs. Several adenoviral vector systems have been developed: first-generation vectors are those with deletion of the E1a and E1b genes, second-generation vectors with deletions of the E1 and another viral gene, and gutless vectors removed all coding sequences for viral proteins. The first- and second-generation vectors are relatively easy to construct and produce. The gutless Ad vectors are substantially superior to any other adenoviral systems for achieving high-level and long-term expression *in vivo*. The recent developed oncolytic adenoviruses can selectively replicate in cancer cells but not in normal cells, allowing the spread of the viruses throughout the tumors. This review focuses on the advantages and limitations of adenovirus vectors and discusses potential strategies for further improvement of vectors for gene therapy.

I. Introduction

Adenoviruses were first isolated by Rowe and colleagues in 1953. These viruses are usually causes of acute, self-limiting upper respiratory tract infections in immunocompetent humans. Adenoviruses may also cause other types of infections, such as eye infections. The viruses are icosahedral particles, which contain a viral capsid that surrounds the viral core (Shenk, 2001). The 36-kb linear dsDNA of the human adenoviral (Ad) genome contains early transcription regions (E1A, E1B, E2, E3, E4) and late transcription regions (L1-5). The Ad genome is intimately associated with viral core proteins and is packaged in the viral capsid, which consists primarily of three proteins: hexon, penton base, and knobbed fibers (Shenk, 2001).

The E1a gene encodes two major regulative produces, 13S and 12S proteins. Both the 12S and 13S proteins bind to members of the retinoblastoma (Rb) tumor suppressor proteins. Sequestration of Rb protein by the E1A proteins frees E2F, which transactivates both viral and cellular promoters containing E2F-responsive

elements. Expression of E1A, without E1B, causes accumulation of the p53 tumor suppressor protein to high levels. The E1B gene encodes the E1B19K and E1B55K proteins which can individually inhibit apoptosis and cooperate with E1A proteins to increase transformation efficiency. The Ad E2 gene encodes three viral proteins: the precursor of the terminal protein (pTP), Ad DNA polymerase and Ad DNA binding protein (DBP). These three proteins are directly involved in viral DNA replication. Initiation of Ad replication occurs via a protein priming mechanism involving the pTP. The Ad DNA polymerase and pTP form a heterodimer which binds specifically to the Ad origin of replication. The Ad DNA binding protein serves to stabilize single-stranded DNA (ssDNA) during replication. E3 encodes products that modulate the response of the host to infection and are primarily directed toward modification of the host immune response. The E4 region comprises at least seven open reading frames (ORFs) whose functions appear disparate. E4 products regulate viral and cellular gene expression, mediate mRNA transport, viral DNA replication and

apoptosis. The viral late genes, L1-L5, are expressed from a common major late promoter and encode the structural proteins for packaging viral DNA into infectious particles.

All viral proteins can be provided *in trans*. The only elements required *in cis* are the two inverted terminal repeats (ITRs, 103 bp for each) that are essential for viral DNA replication, and a 170-bp packaging region that is required for packaging the DNA into virion particles; the packaging region is located at bp 194 to 358 near the left ITR.

Propagation of adenoviruses is dependent on the host cellular machinery for viral protein synthesis and DNA replication. Following infection, viral E1A proteins bind to members of the Rb tumor suppressor family to free E2F. The freed E2F, in turn, binds to the inverted E2F binding sites in the E2a promoter and induces expression of viral E2 genes, which are essential for viral DNA replication. Free E2F induces cells into S-phase by transactivation of cellular promoters containing E2F-responsive elements (Nevins, Leone et al, 1997). However, E2F released by E1A from Rb-E2F may also accumulate active p53 in the nucleus of the cell. p53 accumulated in virus-infected cells may lead to apoptosis, which is presumed to inhibit viral replication before the viral life cycle can be completed (Phillips, Bates et al, 1997; Biederer, Ries et al, 2002). Adenoviral proteins are able to overcome the effects of p53 on apoptosis by some measures. The E1B19K protein is a functional homologue of the anti-apoptotic cellular protein (Bcl-2) and prevents apoptosis by interfering with the actions of the pro-apoptotic proteins, Bak and Bax (Rao et al, 1992; Debbas and White 1993). E1B19K alone can efficiently suppress E1A-induced apoptosis in cancer cells. E1B55K is capable of binding to the amino terminus of p53 and inactivating the effects of p53 (Braithwaite and Jenkins, 1989; Kao et al, 1990). E1B55K can also interact with the Ad E4orf6 protein and degrade p53 protein in adenovirus-infected cells (O'Connor and Hearing 2000). Thus, the major function of Ad E1B proteins is to inhibit p53-dependent and p53-independent apoptosis. Once viral progeny assembly is complete, new viral particles (>1000/cell) are released through cytolysis. Adenoviruses have been developed as powerful vectors that can efficiently transfer genes to a variety of cell types and can be produced with high titer. Ad vectors are particularly attractive for *in vivo* and *ex vivo* gene delivery compared with other viral or non-viral vector systems.

Ad vectors have been developed for use in gene therapy, recombinant viral vaccines, basic science studies, and cancer therapy. The vectors can deliver therapeutic genes into cells to recover the lost function of some genes, to enhance the ability of host immune systems, or to increase the sensitivity of cancer cells to chemotherapeutic drugs. Various Ad vector systems have been developed: first-generation vector with deletions of the E1a and E1b genes (Graham, 1991; Graham and Prevec, 1992, 1995); second-generation vector systems with the E1 regions plus E2a, E2b, or E4 gene deleted (Engelhardt et al, 1994; Gao et al, 1996; Gorziglia et al, 1996; Zhou et al, 1996; Amalfitano et al, 1998; Lusky et al, 1998; Zhou and Beaudet, 2000); the recent gutless or helper-dependent vector systems with removal of all coding sequences for

viral proteins (Kumar-Singh and Chamberlain, 1996; Hardy et al, 1997; Morsy et al, 1998; Steinwaerder et al, 1999; Zhou et al, 2001, 2002a,b;) (**Figure 1**). Adenoviruses are also modified for selective replication in cancer cells with the goal that viral replication, only in cancer cells but not in normal cells, will cause oncolysis.

II. First-generation adenoviral vectors

First-generation Ad vectors are those with deletions of the E1a and E1b genes. Expression cassettes of therapeutic or reporter genes are inserted in the deleted E1 region (Graham, 1991). Removal of the E1 region hampers the transcription of E2 and other viral genes and consequently blocks viral DNA replication. As the E1 products are important for virus replication, vectors with E1 deletion cannot replicate in infected cells. The defective E1 viruses could only be produced in E1 complementing cells, such as 293 cells (Graham et al, 1977; Graham 1987). With deletion of the E1 regions, the vector can carry a transgene of 3 to 4-kb DNA. To increase the carrying capacity in first-generation vectors, the E3 region is also removed in some of the first-generation vectors, because E3 products are not essential for adenovirus replication in cell cultures. Deletions of both E1 and E3 allow the introduction of 5 to 6-kb foreign DNA into first-generation vectors (Graham, 1991). A further increase of the inserted foreign DNA to size 7 or 8 kb is possible with the E1/E3-deleted Ad vectors, but we found it would be very difficult.

The potential problem of the first-generation Ad vectors is pathogenic of the adenovirus backbone (Stratford-Perricaudet et al, 1990; Quantinet et al, 1992; Engelhardt et al, 1993; McElvaney, 1996; Marshall, 1999; van Ginkelet al, 1997). Loss of E1 function reduces, but does not completely eliminate, the ability of the virus to produce other viral proteins (Shenk et al, 1980; Morral et al, 1997; O'Neal et al, 2000). This may account for the toxic effects of first-generation adenovirus vectors after transduction *in vivo*. First-generation vectors generally produce transient expression of therapeutic genes *in vivo*, primarily due to leaky expression of viral genes, leading to immune responses against viral-transduced cells (Mittereder et al, 1994; Yang et al, 1994; Dai et al, 1995). Proteins of the vector particles, once injected into animals, can also elicit innate immune and inflammatory responses (Wolff et al, 1997; Worgall et al, 1997). On the other hand, the products of the therapeutic gene or transgene carried by the vector certainly have the potential to induce host immune responses (Morral, O'Neal et al, 1997). In experimental animals, the viral particle proteins and transgene products may act in an adjuvant-like manner to each other to further stimulate the immune response. The E3 region has the primary function to modify host immune responses; E3 gene may play a critical role in inhibiting inflammatory responses. The expression of E3 products can increase the persistence of transgene expression in some rodent models (Ilan et al, 1997). In addition, the use of tissue-specific promoters, such as a liver-specific promoter that can restrictively express transgenes in a selective tissue, can significantly reduce immune response to the transgene in adenoviral vectors and lead to long-

term expression (Pastore et al, 1999). Thus, elimination of transgene production in professional antigen-presenting cells (dendritic cells and macrophages) may block presentation of foreign proteins to immune systems and decrease immune responses.

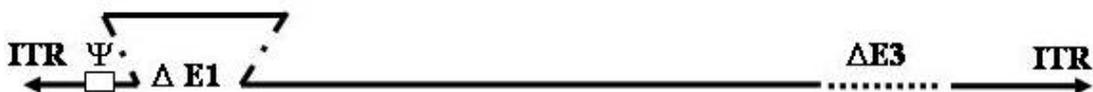
Construction of first-generation Ad vectors are relatively easy and the vectors can be produced with high titers (Graham and Prevec, 1995). The vector system is reliable for efficient transgene delivery in many mammalian cell types and can lead to high-level transient expression. Several commercial kits, Adeno-X™ Expression System (Clontech, Mountain View, CA), AdEasy™ XL System (Stratagene, La Jolla, CA), and ViraPower® Adenoviral Expression System (Invitrogen, San Diego, CA) are available for construction of first-generation Ad vectors. However, these vector systems have space limitations for carrying foreign DNA and are unable to deliver long-term and stable transgene expression *in vivo* due to immune responses to transgene products and/or viral proteins.

III. Second-generation adenoviral vectors

To overcome the limitations of first-generation Ad vectors, second-generation vectors have been developed (Figure 1). Second-generation Ad vectors are generally termed to describe vectors with an additional deletion in other important viral genes based on the backbone of the first-generation E1-deleted vectors. Ad vectors deleted for

E1/E2 or E1/E4 genes could all be called second-generation vectors. As E2 and E4 are required for viral replication, second-generation vectors can only be propagated in cell lines that complement both E1 and E2 or E1 and E4 (Gorziglia et al, 1996; Zhou et al, 1996; Amalfitano et al, 1998; Lusky et al, 1998; Zhou and Beaudet 2000). The replication of adenovirus DNA is dependent on the products of the E2 gene. E2a encodes the viral DNA binding protein and E2b encodes the preterminal protein and DNA polymerase (Shenk, 2001). The E2a product not only is essential for viral DNA replication, this protein also up-regulates transcription from the major late promoter, which controls the synthesis of all viral structural proteins. Thus, E2a-deleted vectors are expected to be safer than first-generation vectors. To increase the titer of E2a-deleted vector, Zhou and Beaudet, applied the tTA-inducible system to enhance E2a promoter activity in complementing cells (Zhou and Beaudet 2000). In this tTA expression system, the tTA binds to the *tet* operators and induces transcription from the promoter in the absence of tetracycline, but not in its presence (Gossen and Bujard 1992; Resnitzky et al, 1994). The E1 and E4 genes play important roles in regulation of viral and cellular gene expression. E1/E4-deleted second-generation vectors are found to have reduced hepatotoxicity and improved stability *in vivo* (Gao et al, 1996; Dedieu et al, 1997). However, one study showed that intravascular delivery of an E1/E4-deleted adenovirus in a patient with

1. First-generation vectors with E1 deletion



2. Second-generation vectors with deletions of E1 and E2 or E4



3. Gutless (helper-dependent) Ad vector



Figure 1. Structures of first-generation, second-generation and gutless adenoviral vectors. The 103-bp inverted terminal repeats (ITR) are located on the left and right termini of the adenoviral genome. The viral DNA packaging signal (Ψ) is within bp 194 to 358 that is required for packing viral DNA into viral particles. The E1 region, including E1a and E1b genes, is deleted in first-generation adenoviral vectors; into this region transgenes can be inserted. In some first-generation adenoviral vectors, the E3 gene is also eliminated to increase the spaces for transgenes. Second-generation vectors are also deleted for E2a or another important viral gene based on the first-generation vector backbone. All viral sequences for encoding viral proteins are removed from gutless Ad vectors.

ornithine transcarbamylase deficiency caused a severe immune system reaction, leading to multiple organ system failure (Marshall, 1999; Raper et al, 2002). In this case, the patient's liver might have not been functioning properly when the adenovirus infused (Marshall, 1999). This incident has led to serious concerns regarding the safety of adenovirus delivered intravascularly.

The second-generation vector systems have provided some degree of improved safety and increased transgene expression in some reports, but they cannot solve virus-associated immunologic problems *in vivo*. Therefore, their ability to produce stable transgene expression has not yet been established (Fang et al, 1996; Morral et al, 1997; Amalfitano, 1999). Construction and preparation of second-generation vectors are relatively difficult compared with first-generation vectors.

IV. Gutless adenoviral vectors

To further reduce the immunogenicity and improve the safety of Ad vectors, the gutless vector system has been developed in which all viral coding sequences are deleted (Figure 1). Production of gutless Ad vectors depends on a helper virus to provide viral gene products *in trans* to support the vector DNA replication and package (Figure 2). Thus, gutless vector is also called helper-dependent Ad vector. The earliest gutless Ad vectors removed only a part of the viral coding sequences and had a mutated packaging region to decrease helper production (Mitani et al, 1995). High levels of helper virus contamination could not be avoided in the final preparations with this system (Kochanek et al, 1996).

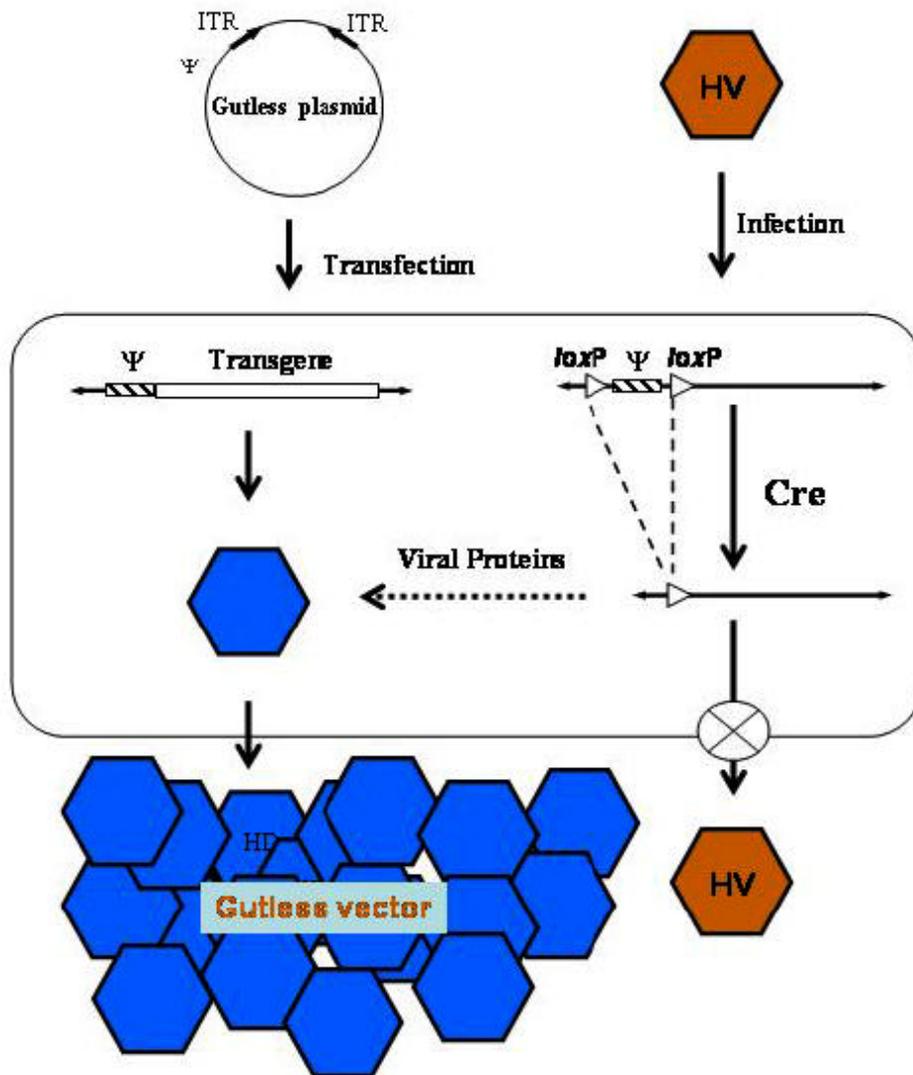


Figure 2. The gutless adenoviral vector system is dependent on Cre/loxP helper adenovirus. Only the inverted terminal repeats (ITR) and the viral DNA packaging signal (Ψ) regions of adenoviral DNA sequences are remained in the gutless vectors. A plasmid containing the entire gutless vector structure can be generated *in vitro*. The gutless DNA then is released from the circular plasmid with a restriction enzyme and transfected into cells that complement for E1 and provide expression of the Cre recombinase. When the helper virus is introduced into the Cre positive cells, the excision of the packaging signal through Cre/loxP in the helper virus renders its DNA unable to be packaged. However, the helper DNA, without its packaging signal region, still can replicate and provide transcripts for all of the necessary proteins for propagation of the gutless vector. The helper viruses (HV) are decreased, but cannot be completely eliminated from the gutless vector production.

An important improvement in the gutless Ad production was the application of the site-specific specific recombination of the bacterial phage P1 Cre recombinase and *loxP* to remove the helper packaging region (Chen et al, 1996; Parks et al, 1996). Cre recombinase expressed in a 293-derived cell line 293Cre4 (Chen et al, 1996) cleaves the packaging signal flanked with *loxP* sites in a helper virus (AdLC8cluc) (Parks et al, 1996). Upon infection of 293Cre4, the packaging signal in the helper virus DNA is excised through the Cre/*loxP* interaction, so that the helper virus DNA without packaging region cannot be packaged into viral particles, but can still produce viral proteins. The viral proteins provided by the helper virus support gutless Ad vectors to replicate, while the helper virus itself is restricted for packaging into viral particles as the packaging signal in it has deleted (**Figure 2**). Although this strategy cannot completely eliminate helper viruses in the final purified gutless vector production, the helper virus contamination decreased to 0.1% or lower levels in gutless Ad vector preparations. For detailed methods regarding construction and preparation of the gutless Ad vector system, recent reviews are available (Ng et al, 2001; Zhou et al, 2002).

Gutless Ad vectors are substantially less toxic than other Ad vectors when high doses are administered intravascularly in mice (Morral et al, 1998; Morsy et al, 1998; Schiedner et al, 1998). These vectors resulted in remarkably long-term expression of transgenes for 1 to 2 years after a single intravascular dose for delivery to the liver in mice and baboons (Kumar-Singh and Chamberlain 1996; Chen et al, 1997; Morsy et al, 1998; Schiedner et al, 1998; Burcin et al, 1999). Low-density lipoprotein receptor gene therapy using gutless vector produced long-term protection against atherosclerosis in a mouse model of familial hypercholesterolemia (Nomura et al, 2004). Gutless Ad vector with liver-restricted expression of human uridine diphospho-glucuronosyl transferase 1A1 achieved lifelong elimination of hyperbilirubinemia in the Gunn rat with negligible chronic toxicity (Toietta et al, 2005). Gutless Ad vector was also used in ex vivo gene delivery of human cystic fibrosis (CF) gene into human sweat glands, showing that the CF gene could be efficiently delivered to, and expressed by, the epithelial cells of sweat glands (Lee et al, 2005). The data obtained suggested that the gutless Ad vector system is one of the most promising viral vector systems for gene therapy.

To investigate acute toxicity after high-dose systemic injection of gutless Ad vectors into nonhuman primates, two baboon were injected with 5.6×10^{12} or 1.1×10^{13} gutless viral particles/kg. In the animal receiving the higher dose, lethal acute toxicity was observed (Brunetti-Pierrri et al, 2004). The acute toxicity was consistent with activation of the innate inflammatory response, suggesting that Ad-mediated acute toxicity is due to viral particles and independent of viral gene expression.

Unlike the first- and second-generation vectors, adequate production of high quality gutless Ad vectors is more difficult. The helper viruses and gutless Ad vectors are both added in cell cultures to coinfect Cre expressing cells. Recombination between the helper viral DNA and the vector DNA within the productive cells may result in

instability of the vector. The inability to produce high quality vectors in large quantity also limits the extensive investigation and application of the vector in gene therapies. Many efforts tried to further improve the gutless Ad vector system by modification of the Cre/*loxP* recombination system or by use of different recombination system, such as FLP/*frt* system (Kumar-Singh and Chamberlain 1996; Hardy et al, 1997; Morsy et al, 1998; Steinwaerder et al, 1999; Ng et al, 2001; Umana et al, 2001; Zhou et al, 2001). However, the current most efficient means of producing gutless Ad vectors are still based on the strategy of deletion of the packaging signal in the helper virus DNA. A major breakthrough in gutless vector production is still needed before this type of vector may be ready for clinical applications.

V. Oncolytic adenoviruses

Oncolytic adenoviruses are viruses that can selectively replicate in cancer cells but not in normal cells. There are several recent review articles (Mullen and Tanabe 2002; Haviv and Curiel 2003; Nettelbeck 2003; Kanerva and Hemminki 2004; Kanerva and Hemminki 2005). Viral replication kills cancer cells and causes their disintegration, releasing newly made viruses. The primary advantage of replication-competent oncolytic viruses is that viral replication will amplify the initial input dose, allowing the spread of vector throughout the target tissues. Certain oncolytic viruses can also generate some proteins that are directly cytotoxic to the host cells (Shtrichman and Kleinberger 1998; Tollefson et al, 1998).

Adenovirus dl1520 (ONYX-015), deleted for E1B55K (Barker and Berk, 1987), is able to replicate in cancer cells lacking active p53, but unable to replicate in normal cells with functional p53 (Bischoff et al, 1996). However, work by several other laboratories found that a variety of tumor cell lines, regardless of their p53 statuses, allow efficient replication of dl1520 and other E1B55K-mutated viruses (Dix, Edwards et al. 2001; Geoerger, Grill et al. 2002; Hobom and Dobbstein 2004; O'Shea, Johnson et al. 2004). dl1520 has been applied in multiple clinical trials and the results have been reviewed recently (Kirm 2001; Nemunaitis and Edelman 2002; Reid, Warren et al, 2002; Dobbstein 2004; McCormick 2003; Post, Khuri et al, 2003). dl1520 has shown promise and safety in Phase I and II clinical trials in cancer patients following administration via multiple routes. In combination with chemotherapies, intratumoral injection of dl1520 into recurrent head and neck cancers was encouraging compared with virotherapy or chemotherapy alone in the parallel studies (Kirm et al, 1998; Nemunaitis et al, 2000; Nemunaitis and O'Brien, 2002).

Adenovirus E1A is the first transcription unit to be expressed upon infection of cells. There are two major E1A products: the 12S and 13S proteins. The E1A 13S protein contains three conserved regions (CR1, CR2, and CR3), and the E1A 12S protein contains two of the conserved regions (CR1 and CR2). Both E1A 12S and 13S proteins bind to the pRb protein through CR1 and CR2 to release E2F from the E2F-pRB complex (Bagchi, Raychaudhuri et al, 1990; Bandara and La Thangue 1991). As viral E1A CR2 is involved in binding with pRb protein,

the CR2 role for pRb binding may not be required in cancer cells with mutated or disrupted pRb function. Ad ϕ 24 with deletion of a 24-bp region in the CR2 was constructed and tested with mutated and wild-type pRb cells (Fueyo et al, 2000). The study showed that Ad ϕ 24 replicated in and lysed most of the pRb-mutated human glioma cells and inhibited tumor growth in nude mice.

Ad *E1a* gene is the first transcription unit to be expressed upon infection and its products are critical in regulation of other viral gene expression. One of the strategies for targeting replication of oncolytic adenovirus in tumor cells is to regulate the expression of *E1a* gene by using tumor-specific promoters that are highly active in cancer cells but not in normal cells. This strategy was used to target adenoviruses to prostate cancer cells by using prostate-specific antigen (PSA) promoter (Rodriguez et al, 1997; Yu et al, 1999). PSA, a widely used marker for the diagnosis of prostate cancer, is highly expressed in most prostate cancer cells. *E1A* was expressed at high levels in PSA positive prostate cancer cells but not in PSA negative prostate or other cancer cells after CV706 infection. A phase I trial of CV706 for the treatment of locally recurrent prostate cancer demonstrated that intraprostatic delivery of CV706 was safe at high doses and treatment resulted in strong reduction of serum PSA levels (DeWeese et al, 2001). Other cancer specific promoters are also applied for cancer gene therapy (Mullen and Tanabe 2002; Haviv and Curiel 2003; Nettelbeck 2003; Kanerva and Hemminki 2004; Kanerva and Hemminki 2005).

VI. Discussion

Several Ad vector systems are available for gene therapy, the first-generation, second-generation, gutless Ad vectors, and recent oncolytic viruses. The first-generation and second-generation vectors cannot lead to long-term transgene expression. However, these vectors can efficiently infect various types of cells and are relatively easy to construct and produce. Thus, they are broadly applied some applications, such as vaccination and cancer gene therapy, that do not require extended transgene expression. The gutless Ad vectors are substantially superior to any other viral system for achieving high level and long-term expression *in vivo*. The major weakness of the current approaches in production of gutless Ad vector is the requirement for three components: a specialized cell line, a helper virus, and the gutless Ad vector. With the current system, it is difficult to produce high quality gutless Ad vectors in large quantity and viral DNA rearrangements involving the helper and the vector was observed (unpublished data, Zhou H). Removing the helper virus from the gutless Ad vector system is necessary before the vector could possibly be applied in clinical treatments. A future direction should be development of a packaging cell line with inducible production of all the required viral proteins for gutless Ad vector production could eliminate the need for helper virus. Oncolytic adenoviruses have the potential to destroy cancer cells via selective replication. There are two types of oncolytic approaches: viruses with mutations in genes that are required for viral life cycle in normal cells (such

as dl1520) and viruses with their essential genes controlled by cancer specific promoters (such as CV706). It appears that combination of these two strategies will improve the safety and efficacy.

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H. Sam Zhou

