Review

Development and Evaluation of a Novel Gene Delivery Vehicle Composed of Adenovirus Serotype 35

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The capacity of gene delivery vehicles is considered to be a critical factor determining the success of gene therapy. To date, various types of gene delivery vehicle have been developed. Among them, recombinant adenovirus (Ad) vectors have potential that has favored their worldwide use *in vitro* and *in vivo*. Conventional Ad vectors are composed of subgroup C Ad serotype 5 (Ad5), although it has been clarified that the drawbacks of Ad5 vectors are a high seroprevalence of Ad5 in adults and low transduction efficiencies in cells lacking the primary receptor for Ad5, coxsackievirus and adenovirus receptor. To overcome these problems, we developed a novel Ad vector fully composed of Ad serotype 35 (Ad35). Ad35 vectors show a wide tropism for human cells because Ad35 binds to human CD46, which is ubiquitously expressed on almost all human cells, as a primary receptor. In addition, anti-Ad5 antibodies do not inhibit Ad35 vector-mediated transduction and the seroprevalence of Ad35 in adults is lower than that of Ad5. This paper reviews our studies on the development and evaluation of Ad35 vectors. Ad vectors derived from other Ad serotypes different from Ad5, including Ad35, are expected to be gene delivery vehicles alternative to conventional Ad5 vectors.

Key words adenovirus vector; gene delivery; CD46; serotype; neutralizing antibody

INTRODUCTION

Gene therapy, in which diseases are treated by introducing foreign genes into cells, has been promising for the treatment of various types of disease, including hereditary diseases, cancer, and infectious diseases. More than 1300 clinical protocols for gene therapy have been performed worldwide. In addition, gene delivery technology is now essential for basic studies, such as analysis of gene function and genetic manipulation of cells including induced pluripotent stem (iPS) cells.^{1,2)} In both gene therapy and basic studies, transduction efficiencies of gene delivery vehicles are crucial, and various types of gene delivery vehicle, including retrovirus vectors, adenovirus (Ad) vectors, and lentivirus vectors, have been developed. Among gene delivery vectors, Ad vectors are considered to be very promising because they are relatively easy to construct, can be produced at high titer, and have high transduction efficiencies. In addition, Ad vectors do not cause genotoxicities that are induced by the insertion of viral DNA into the host chromosome. Conventional Ad vectors are composed of subgroup C Ad serotype 5 (Ad5), along with Ad serotype 2, because they are the most thoroughly studied serotypes among the 51 human Ads (Table 1).³⁾ Conventional Ad5 vectors have several advantages described above, although recent studies, including clinical studies, have revealed some drawbacks of Ad5 vectors (Fig. 1). First, Ad5 vectors mediate poor transduction in cells lacking the expression of the primary receptor for Ad5, the coxsackievirus and adenovirus receptor (CAR). Unfortunately, important target cells for gene therapy, such as dendritic cells, hematopoietic stem cells, and malignant tumor cells, express insufficient levels of CAR, leading to resistance to Ad5 vector-mediated transduction. The second hurdle is the inhibition in adults of Ad5 vector-mediated transduction by pre-existing anti-Ad5 immunity, which is induced as a result of natural Ad5 infection.^{4–6)} The transduction efficiencies and therapeutic effects of Ad5 vectors are largely decreased by

neutralizing anti-Ad5 antibodies, even when Ad5 vectors are locally administered.^{7–9)} In addition, the liver toxicity of Ad5 vectors upon readministration may be increased in the presence of anti-Ad5 antibodies.¹⁰⁾

Table 1. Human Adenovirus Serotypes

Subgroup	Serotypes	Receptor ^{a)}
A	12, 18, 31	CAR
B1	3, 7, 16, 21, 50	CD46
B2	11, 14, 34, 35	CD46
C	1, 2, 5, 6	CAR
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	CAR
E	4	CAR
F	40, 41	CAR

CAR; coxsackievirus and adenovirus receptor. a) Some Ad serotypes recognize other receptors different from CAR and CD46.

Fig. 1. Schematic Diagram of the Properties of Ad5 and Ad35 Vectors

Subgroup C Ad5 vectors infect cells *via* interaction with CAR. Subgroup B Ad35 vectors recognize human CD46 as a receptor. Anti-Ad5 antibodies inhibit infection of Ad5 vectors, but not of Ad35 vectors.

To overcome these problems, we developed a novel type of Ad vector that is fully composed of subgroup B Ad serotype 35 (Ad35).^{11,12} Ad35 is an uncommon subgroup B Ad that has been often isolated from immunocompromised hosts. Ad35 was originally identified from the lungs and kidneys of a renal transplant recipient.¹³ In addition, there have been reports of Ad35 isolates from bone marrow transplant patients and patients with AIDS-related complex.^{14,15} Ad35 has promising potential that can overcome the drawbacks of Ad5 vectors as a platform for gene delivery vehicles (Fig. 1). First, another molecule different from CAR serves as a receptor for Ad35. (Receptor(s) for Ad35 were not unknown when we started the study of Ad35 vectors. It was revealed in 2003 that human CD46 is a receptor for subgroup B Ads.)^{16–18} Second, the seroprevalence of Ad35 in adults has been found to be significantly lower than that of Ad5.^{4,5} Third, anti-Ad5 immunity does not inhibit infection of Ad35. Here I review our recent studies on the development and evaluation of Ad35 vectors.

DEVELOPMENT OF AD35 VECTORS

Ads have approximately 35–36-kb double-stranded DNA as a genome, which is roughly classified into early genes (E1–E4) and late genes (L1–L5). Early genes and late genes are mainly involved in the replication of the viral genome and synthesis of capsid proteins, respectively. To develop a replication-incompetent Ad vector, the E1 region, which is located at the left end of the genome, should be removed because the E1 gene products induce the transcription of almost all other viral genes. In conventional replication-incompetent Ad5 vectors, the E1 region is deleted and a transgene cassette is inserted into the E1-deleted region. Conventional Ad5 vectors cannot replicate in normal cells due to the absence of the E1 products, and Ad5 vectors are propagated in 293 cells, which are able to provide the E1 gene products of Ad5 *in trans*.

The E1 region should be removed to develop a replication-incompetent Ad35 vector in a similar way as with Ad5 vectors; however, sequence information on the Ad35 genome was not available when we started the study of Ad35 vectors. Therefore we first constructed the restriction endonuclease cleavage map of the Ad35 genome by digesting the Ad35 genome with restriction endonucleases.¹¹ After the construction of a restriction map of the Ad35 genome, the whole Ad35 genome was cloned into a plasmid, and the fragment (bp 365–2912) that contained E1A and a part of E1B was removed. Subsequently, a transgene expression cassette was inserted into the E1-deleted region, resulting in a vector plasmid for the replication-incompetent Ad35 vector. Almost all the E1 region is removed in conventional Ad5 vectors, but the Ad35 vectors we developed retain a part of the E1B region, which is considered to contain a promoter sequence of pIX. Unlike Ad5, the promoter region of pIX is incorporated into the E1B region of Ad35.¹⁹ Complete deletion of the E1 region of Ad35 impairs pIX expression, resulting in a reduction in virus particle stability. pIX is a minor protein stabilizing hexon–hexon interactions. We should pay attention to the deletion size of the Ad35 E1 region for the construction of Ad35 vectors.

For the production of Ad35 vectors with both E1A and

E1B deleted, the Ad35 vector plasmid was digested with *Sbf*I to release the Ad35 vector genome from the plasmid backbone and subsequently transfected in VK10-9 cells (kindly provided by Dr. V. Krougliak, Ontario, Canada), which are 293 cells expressing the E4 proteins of Ad5 as well as the E1 proteins.²⁰ Replication-incompetent Ad35 vectors did not replicate in conventional 293 cells. VK10-9 cells successfully supported the replication of Ad35 vectors, and the final virus yield from seven plates of 150-mm dishes was approximately 1.5 ml of virus at 10¹¹ vector particle (VP)/ml, which was slightly lower than the yield in the case of Ad5 vector preparation.¹¹ It has been demonstrated that Ad35 vectors are also able to propagate in packaging cell lines expressing both Ad5 E1 proteins and Ad35 E1B-55K protein.^{4,21,22} The Ad5 E1B-55K protein has been reported to form a complex with the Ad5 E4 open-reading frame (ORF)6 protein and to increase selective export of late viral mRNA.^{23,24} These results suggest that E1B-55K and E4 ORF6 proteins should be derived from the same subgroup for virus replication. These packaging cell lines described above provide an important advantage: replication-competent Ad35 does not appear in culture by homologous recombination because the homologous region between the genomic DNA of Ad35 vectors and the complement cell lines is not found.

Ad35 vectors containing a transgene expression cassette have often been constructed by homologous recombination in packaging cell lines or *Escherichia coli*; however, these methods are inefficient and difficult to perform. We have applied an improved *in vitro* ligation method, which was developed by Mizuguchi and Kay,^{25,26} to the construction of Ad35 vectors to obtain Ad35 vectors more rapidly and easily (Fig. 2).²⁷ We can produce replication-incompetent Ad35 vectors as easily and efficiently as Ad5 vectors using this method.

IN VITRO TRANSDUCTION PROPERTIES OF AD35 VECTORS

Expression profiles of virus receptors largely determine the tropism of viruses, including Ad vectors. As described above, the primary receptor for Ad5 vectors, CAR, is broadly expressed in various types of cells, including hepatocytes and epithelial cells, leading to efficient transduction in these cells. In contrast, subgroup B Ads, including Ad35, have long been known to utilize an unknown receptor(s) other than CAR.¹⁸ The unidentified receptors had been expected to be broadly expressed because Ad35 vectors and fiber-chimeric Ad5 vectors containing the fiber protein of Ad35 exhibited a broad tropism, including CAR-deficient cells, *in vitro*.^{12,28} Thereafter, in 2003, human CD46 was identified as a receptor for subgroup B Ads.^{16,17} Two years later we succeeded in the development of replication-incompetent Ad35 vectors.

Human CD46 is a single-chain type I transmembrane glycoprotein with a molecular mass of 45–70 kDa and is largely involved in protecting autologous cells from complement-mediated damage by serving as a cofactor for factor I-mediated inactivation of C3b and C4b, leading to blockade of the complement cascade.^{29,30} Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine-threonine-proline-rich region, a short region of unknown function, a transmembrane domain, and a carboxy-terminal cytoplas-

Fig. 2. Construction of Replication-Incompetent Ad35 Vectors Using an Improved *in Vitro* Ligation Method, Which Was Originally Developed by Mizuguchi and Kay^{25,26}

Fig. 3. Ad35 Vector-Mediated Transduction in CHO Cells Expressing CD46 Mutants Lacking SCRs

(A) A schematic diagram of human CD46. (B) Transgene expression levels by Ad35 vectors in CHO cells expressing the CD46 mutants. The cells were transduced with Ad35 vectors expressing firefly luciferase at 3000 VP/cell for 1.5 h. Following a 48 h-incubation, the luciferase productions in the cells were measured in the chemiluminescence assay. The data were normalized to the luciferase production in parental CHO cells. Cited from Sakurai *et al.*³¹ The asterisks indicate the level of significance ($p < 0.005$ [double asterisk] for comparison with CHO-CD46 cells).

mic domain (Fig. 3A). We demonstrated using anti-CD46 antibodies and mutant CD46-expressing cells that SCR1 and SCR2 contain the binding site for Ad35 among these components (Fig. 3B).³¹ Recognition of SCR1 and SCR2 by Ad35 vectors would be convenient for infection of Ad35 because SCR1 and SCR2 are located on the upper region of human CD46, leading to the decrease in electrostatic repulsion between the virus capsid and acidic cell surface proteins and the increase in attachment of Ad35 to the cell surface. Human CD46 has a unique property in its expression profile. Human CD46 is expressed on almost all human cells except for erythrocytes, leading to the broad tropism of Ad35 vectors. Several groups reported that Ad35 vectors efficiently transduce various types of human cell, including human bone marrow CD34⁺ cells, dendritic cells, synovocytes, and smooth muscle cells, which are refractory to Ad5 vectors due to their lack of CAR expression.^{4,11,12,21} In addition, the expression of human CD46 is often upregulated in cancer cells compared with normal cells,³² suggesting that cancer cells overexpressing human CD46 would be a suitable target for Ad35 vectors. Brouwer *et al.* demonstrated that Ad35 vectors

mediated more efficient transduction in primary glioma cells than conventional Ad5 vectors.³³ CD46 expression levels are an important determinant of the transduction efficiencies of Ad35 vectors. On the other hand, Ad35 vectors do not transduce rodent cells because expression of rodent CD46 is limited to the testis and there is low homology between human and rodent CD46.^{34,35}

Human CD46 is also well known to serve as a receptor for several pathogens, including measles virus and human herpesvirus type 6.³⁶ These pathogens bind to distinct sites of human CD46; however, surface expression levels of human CD46 are largely downregulated after infection with these pathogens. We found that infection of Ad35 vectors also decreased the surface expression levels of human CD46 on human peripheral blood cells (Fig. 4) and leukemia cell lines, although nonleukemia cells (A549, HeLa) did not exhibit the downregulation of human CD46.³⁷ These results suggest that we should exercise caution in the use of Ad35 vectors. Previous studies demonstrated that downregulation of human CD46 leads to an increase in the susceptibility of cells to complement-mediated cell lysis.^{38,39}

IN VIVO TRANSDUCTION PROPERTIES OF AD35 VECTORS

The *in vivo* tropism of Ad vectors is more complicated and differs greatly from that *in vitro*. The ablation of CAR binding by amino acid substitution in the fiber knob of Ad5 did not decrease *in vivo* transduction, although *in vitro* transduction efficiencies were markedly decreased, indicating that CAR does not play an important role in the *in vivo* tropism of Ad5 vectors.^{40,41} Both CAR and αv integrin binding should be ablated to decrease the transduction efficiencies of Ad5 vectors in the organs.^{42,43} In addition, the lysine-lysine-threonine-lysine (KKTK) motif in the fiber shaft of Ad5 was demonstrated to be important for binding to heparan sulfate.⁴⁴ Recent studies have also revealed that coagulation factor X (FX) directly binds to the Ad5 hexon and that FX

forms a bridge between the Ad5 hexon and heparan sulfate on the cell surface.^{45,46}

To examine the *in vivo* transduction properties of Ad35 vectors, we performed *in vivo* transduction experiments with Ad35 vectors using wild-type mice.¹² Intravenous administration of Ad35 vectors resulted in poor transduction in the organs of mice. The transduction efficiencies of Ad35 vectors in the liver were four log orders lower than those of Ad5 vectors. We hypothesized that the poor transduction efficiencies of Ad35 vectors in wild-type mice would be due to the lack of CD46 expression in wild-type mice. Therefore Ad35 vectors were intravenously or intraperitoneally administered to human CD46-transgenic (hCD46TG) mice (kindly provided by Dr. M. Okabe, Osaka University, Osaka, Japan), which ubiquitously express human CD46 in a pattern similar to humans.²² Ad35 vector-mediated transgene expression was higher in hCD46TG mice than in wild-type mice; however, the transgene expression levels of Ad35 vectors in hCD46TG mice were still much lower than those of Ad5 vectors in wild-type mice. In addition, mesothelial cells on the surface of organs were mainly transduced with intraperitoneally injected Ad35 vectors, although intraperitoneal administration of Ad35 vectors resulted in higher transduction efficiencies in the organs than intravenous administration. We further examined the transduction properties of Ad35 vectors using nonhuman primates, *i.e.*, cynomolgus monkeys.⁴⁷ The CD46 of nonhuman primates is ubiquitously expressed on almost all types of cells and shows high homology to human CD46. Intravenous administration of Ad35 vectors resulted in poor transduction in the organs of cynomolgus monkeys, as seen in hCD46TG mice, although Ad5 vectors mediated successful transduction mainly in the liver and spleen (Fig. 5). These results indicate that Ad35 vectors cannot efficiently transduce organs in spite of CD46 expression when introduced into the blood circulation. It remains to be elucidated why Ad35 vectors poorly transduce organs following intra-

Fig. 4. Downregulation of Human CD46 from the Cell Surface of Peripheral Blood Mononuclear Cells (PBMCs) after Injection with Ad35 Vectors

PBMCs were incubated with Ad35 vectors at 10000 VP/cell for up to 48 h. Cells were harvested at the indicated time points and stained with monoclonal anti-human CD46 antibody after fixation. The expression levels of human CD46 on the cell surface were determined using flow cytometry. The percentages of surface CD46 downregulation were calculated as follows: $\text{CD46 downregulation} = 100 - (100 \times \text{MFI of CD46 in infected cells}) / (\text{MFI of CD46 in uninfected cells})$, where MFI = mean fluorescence intensity. Cited from Sakurai *et al.*³⁷

Fig. 5. Ad Vector-Mediated Transgene Expression in Cynomolgus Monkeys after Systemic Injection

Ad5 or Ad35 vectors expressing β -galactosidase were intravenously administered to cynomolgus monkeys at 2×10^{12} VP/kg through the saphenous vein. Four days after administration, the organs were collected, and X-gal staining of tissue sections was performed using routine methods. Cited from Sakurai *et al.*⁴⁷

Fig. 6. Transduction Efficiencies of Ad5 and Ad35 Vectors in Human Bone Marrow-Derived CD34⁺ Cells

Human CD34⁺ cells were suspended in medium containing the cytokine cocktail. The cells were transduced with Ad5 vectors or Ad35 vectors expressing enhanced green fluorescence protein (GFP) at 300 plaque forming units (PFU)/cell 16–18 h after seeding. The culture medium was added 1.5 h after transduction. The cells were incubated without removal of Ad vectors. Following 48-h incubation, the cells were recovered, washed, and subjected to flow cytometric analysis.

venous administration; however, there are two possible explanations for the low transduction activity of Ad35 vectors. First, Ad35 vectors might be more susceptible than Ad5 vectors to degradation in the blood or in intracellular compartments such as endosomes/lysosomes. Second, Ad35 vectors might not be able to gain access to CD46 after intravenous administration. CD46 is mainly expressed on the basolateral sides of cells,^{48,49} making it inaccessible to Ad35 vectors. Ad35 vectors that are not able to bind to CD46 on the cell surface would be phagocytosed into phagocytic cells, leading to degradation.

The low transduction activities of Ad35 vectors *via* intravenous delivery could offer another potential advantage in their use, namely, that locally administered Ad35 vectors would not cause unwanted side effects in organs other than the targeted organs when draining from injected sites into the blood circulation. This is in contrast to Ad5 vectors, which, after local injection, have been shown to drain into the systemic circulation and cause unwanted side effects in organs.^{50,51} Therefore Ad35 vectors were locally administered into various organs of cynomolgus monkeys. Direct injection of Ad35 vectors in the organs resulted in efficient transduction in the area around the injected sites. Significant transgene expression was not obtained in the organs where Ad35 vectors were not administered. These results indicate that Ad35 vectors would be suitable for transduction *via* local administration into organs.

Transduction experiments using Ad35 vectors in cynomolgus monkeys *via* systemic injection also revealed that Ad35 vectors induced less severe tissue damage and inflammatory responses compared with Ad5 vectors.⁴⁷ Previous studies also demonstrated that Ad35 vectors are less immunogenic than Ad5 vectors.^{7,9} In contrast, intravascular injection of Ad5 vectors rapidly and systemically induces inflammatory responses, leading to tissue damage, and this can be fatal to the host.^{52–55} These safety profiles of Ad35 vectors could provide major advantages in their clinical use.

TRANSDUCTION IN HUMAN BONE MARROW-DERIVED CD34⁺ CELLS

Hematopoietic cells, including hematopoietic stem cells (HSCs), are important targets for gene therapy. In particular, efficient transduction into HSCs would afford the opportunity to treat a number of diseases that result from abnormal

blood cell function due to their potential for self-renewal and multilineage differentiation into all mature blood cells. However, these cells are refractory to Ad5 vectors due to the lack of CAR expression.^{56,57} In contrast, high affinity of Ad35 to blood cells, including human CD34⁺ cells, which contain an HSC population, were known before the development of recombinant Ad35 vectors.^{58,59} Therefore we examined the transduction profiles of Ad35 vectors in human bone marrow-derived CD34⁺ cells. Ad35 vectors mediated higher transduction efficiencies in the human CD34⁺ cells than Ad5 vectors (Fig. 6).¹¹ More than 50% of the CD34⁺ cells exhibited transgene expression with Ad35 vectors, but the CD34⁺ cells were almost completely refractory to Ad5 vectors. Transduction with Ad35 vectors did not exert apparent toxicities on the CD34⁺ cells. Furthermore, Ad35 vectors efficiently transduced the CD34⁺CD38[−] cells and CD34⁺AC133⁺ cells, which are more primitive subsets among CD34⁺ cells.²⁷ Ad35 vector-mediated transgene-expressing cells showed a colony-formation capacity similar to that of nontransduced cells. These results suggest that Ad35 vectors can transduce hematopoietic progenitors that have the abilities to proliferate and differentiate following transduction and that transduction with Ad35 vectors does not inhibit the proliferation and differentiation of hematopoietic progenitors. Now we are trying to manipulate CD34⁺ cells by introducing functional genes using Ad35 vectors to differentiate CD34⁺ cells into specific lineages or to promote self-renewal of CD34⁺ cells.

CONCLUSIONS

Based on our study of and recent progress in Ad vectors fully composed of subgroup B Ad35, Ad35 vectors have the potential to overcome the drawbacks of conventional Ad5 vectors. However, some problems remain to be resolved before the clinical application of Ad35 vectors. First, the plaque-forming unit (PFU)-to-particle ratios of Ad35 vectors are often lower than those of Ad5 vectors. Both infectious and noninfectious virus particles induce immune responses after *in vivo* application, indicating that we should prepare Ad35 vectors at high PFU-to-particle ratios to circumvent Ad vector-induced immune responses. Second, the fundamental properties of Ad35 are poorly understood. Characterization of Ad35 would provide valuable information to predict the transduction profiles of Ad35 vectors. Further improvements

and evaluation are necessary for their useful clinical applications.

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