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Design and synthesis of a Tat-related gene transporter: A tool for carrying the adenovirus vector into cells

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Abstract—A Tat-related peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide, designed to transport an Adenovirus vector (Ad) into cells, was synthesized. The synthetic peptide was conjugated to Ad, which potentially can act as an efficient carrier of heterologous genes into cells. The Tat-related peptide was synthesized using the solid phase method and then was coupled to the heterofunctional cross-linking reagent, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester. The resulting peptide-succinimidohexanoic acid *N*-hydroxysuccinimide ester was conjugated to Ad containing the luciferase gene. B16BL6 cells infected with the peptide-conjugated Ad luciferase gene construct exhibit a 50-fold greater luciferase activity than B16BL6 cells infected with wild-type Ad containing the luciferase gene.

Gene therapy has attracted much attention as a potential clinical treatment/cure for intractable diseases.[1] A key to the successful implementation of gene therapy protocols is the design of the transgenesis vector. Adenovirus vectors (Ad) are often used as transport agents during gene therapy experiments and trials since they exhibit suitable transduction and gene-expression properties; but for routine clinical procedures, more efficient transfer vectors need to be developed. Previously, we showed that an Arg-Gly-Asp(RGD)-related peptide that binds to integrin functions as an efficient auxiliary transporter of Ad.[2] The RGD-related peptide, when covalently bound to Ad, transports Ad into dendritic cells via interaction with integrins. For this report, a different type of Ad auxiliary transporter was designed, synthesized, and shown to greatly increase the amount of Ad (containing the luciferase gene) transferred into cells.

The human immunodeficiency virus (HIV)-1 protein, Tat, is a transcriptional activator of HIV and can cross both the plasma and nuclear membranes. Tat contains 86 amino acids, but its translocation activity is associated with the peptide sequence, Tat(48–60), (GlyArgLysLysArgArgGlnArgArgArgProProGln: GRKKRRQRRRPPQ).[3] Futaki et al. reported that certain synthetic arginine-rich peptides can readily cross cell membranes and that the optimal number of arginines required for efficient translocation is approximately eight.[4] We designed the peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide (Ac-GRRRRRQRRRPPQGC-NH_2), to be an efficient auxiliary transporter of Ad. Since Futaki et al.[4] reported that the number of arginine residues correlates with translocation ability, the sequence, Ac-GRRRRRQRRRPPQGC-NH_2, was designed so that the two lysines found in Tat(48–60) were replaced with arginines. A C-terminal cysteine was added so that the peptide could be linked to Ad through the heterofunctional cross-linking reagent 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS),[5] which reacts with amine and sulfhydryl moieties (Fig. 1). The peptide was synthesized using an Applied Biosystems Peptide Synthesizer 433A-1. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids [Fmoc-Gly-OH; Fmoc-Pro-OH; N^{α}-Fmoc-N^{G}-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony-

Keywords: Adenovirus vector; Tat; Tat-peptide; Peptide synthesis.
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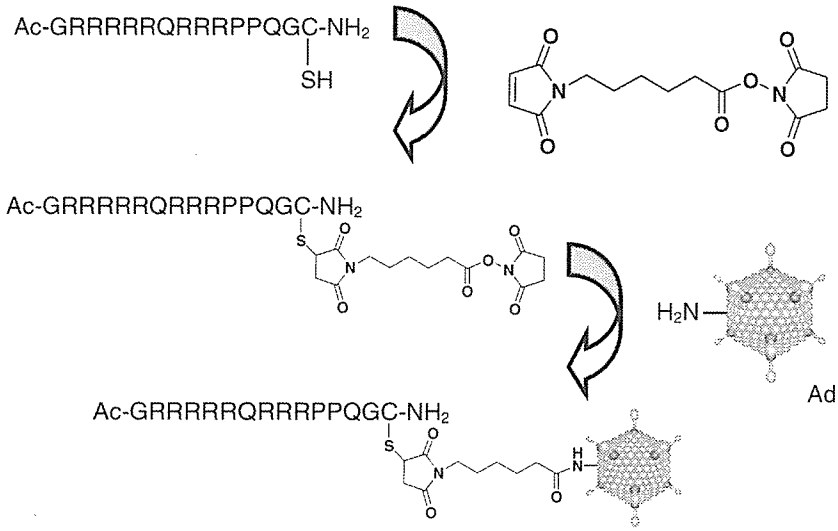

Figure 1. Synthesis of a peptide–Ad conjugate that acts as an efficient heterologous gene transporter.

larginine: Fmoc-Arg(Pbf)-OH; N^{α}-Fmoc-*S*-tritylcysteine: Fmoc-Cys(Trt)-OH; and Fmoc-Gln(Trt)-OH] were coupled in a stepwise manner to Rink amide resin[6] (PE Biosystems. Amino content: 0.67 mequiv/g, 379 mg, 0.25 mmol) using the coupling reagent, 2-(1-*H*-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[7] in *N*-methylpyrrolidone (NMP). After each coupling step, the Fmoc group was removed using 20% piperidine/NMP. The synthetic Fmoc-Gly-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Pro-Pro-Gln(Trt)-Gly-Cys(Trt)-Rink amide resin was treated with 20% piperidine/NMP and then treated with acetic anhydride. The peptide was cleaved from the resin with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (95:2.5:2.5). The resulting crude peptide, (Ac-GRR RRRQRRRPPQGC-NH_2, 410 mg yield), was purified using RP-HPLC.[8] The HPLC profile of the crude peptide mixture is shown in Figure 2 The yield of the purified peptide was 202 mg (28% as calculated from the amino content of the used resin).

The purified peptide (40 mg, 14 μmol), dissolved in PBS (pH 7.2, 500 μL), and the heterofunctional cross-linkage reagent (MHS: 4.3 mg, 14 μmol), dissolved in dimethylsulfoxide (DMSO, 10 μL), were combined and then stirred for 0.5 h. We attempted to purify the product, Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 (SHS: 6-succinimidohexanoic acid *N*-hydroxysuccinimide ester), using HPLC, but could not—the *N*-hydroxysuccinimide ester hydrolyzes easily in water; therefore, the reaction product mixture was frozen immediately and kept at −80 °C until needed. While gently stirring, Ad-Luc, whose chromosome encodes the heterologous luciferase gene, was reacted with Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 at 37 °C for 45 min. To test the relative transduction efficiency of the peptide–Ad conjugate [Tatpep-(Ad-Luc)],

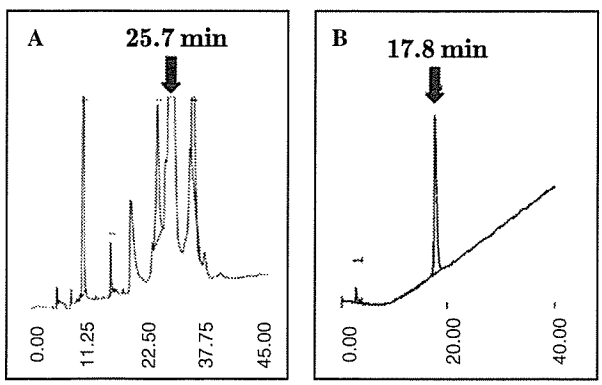

Figure 2. HPLC profile of synthetic crude Ac-GRRRRRQ-RRRPPQGC-NH_2. (A) Preparative HPLC of crude synthetic peptide. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10–70% CH_3CN over the course of 60 min. The absorbance was measured at 220 nm. (B) Analytical HPLC of purified sample. Column: Inertsil ODS-3 (4.6 × 250 mm). Flow rate: 1 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 5–20% CH_3CN over the course of 40 min. The absorbance was measured at 220 nm.

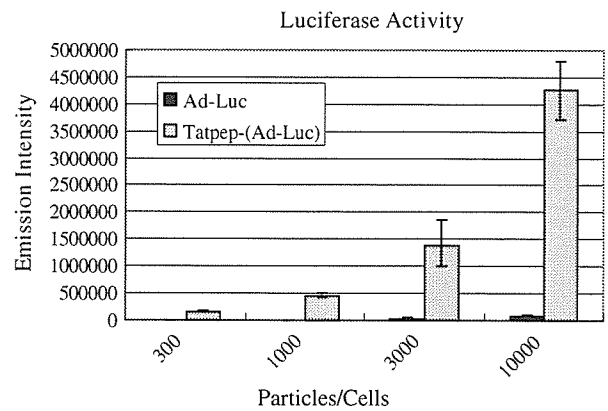

Figure 3. Transduction efficiency of Ad-Luc and Tatpep-(Ad-Luc) into B16BL6 cells Cells (2×10^4) were incubated with 300, 1000, 3000 or 10,000 particles/cell of Ad-Luc (solid bars) or Tatpep-(Ad-Luc) (gray bars). Luciferase activity, which was determined using a Luciferase Assay System Kit (Promega, USA) and a Microlumat Plus LB96 (Perkin-Elmer, USA) after lysing the cells with Luciferase Cell Culture Lysis Reagent (Promega, USA), was measured at the end of a 24 h incubation. The bars report the mean relative unit of light per well ± SD ($n = 3$).

B16BL6 cells were incubated with it or with Ad-Luc for 24 h, at which time luciferase activity was measured (Fig. 3). B16BL6 cells were used because the Coxackie-adenovirus receptor, which transports Ad across the plasma membrane, is nearly absent.[9] At concentrations of 300 and 1000 particles/cell, cells that were exposed to Ad-Luc did not glow, while those exposed to Tatpep-(Ad-Luc) construct clearly did. Ad-Luc infected cells glowed weakly at doses of 3000 and 10,000 particles/cell, while Tatpep-(Ad-Luc) exhibited strong luciferase activity at the same concentrations. The transduction activity of Tatpep-(Ad-Luc) is about 50-fold greater than that of Ad-Luc—a remarkable finding.

In summary, we designed the peptide, Ac-GRRRRRQ-RRRPPQGC-NH_2, to be an efficient auxiliary transporter of Ad into cells. Ad, when covalently bound to this synthetic peptide, exhibits a transduction ability 50-fold greater than does Ad alone. This modified Ad is a promising experimental tool for transduction studies.

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reviews

Adenovirus Vector-Mediated Gene Transfer into Stem Cells

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Abstract: Stem cells, including embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), are defined by their capacity for self-renewal and multilineage differentiation. Efficient gene transfer into stem cells is essential for the basic research in developmental biology and for therapeutic applications in gene-modified regenerative medicine. Adenovirus (Ad) vectors, based on Ad type 5, can efficiently and transiently introduce the exogenous gene into many cell types via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSCs and HSCs, cannot be efficiently transduced with conventional Ad vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified Ad vectors and an Ad vector based on another serotype of Ad have been developed. Here, we review the advances in the development of Ad vectors suitable for stem cells and discuss their application in basic biology and clinical medicine.

Keywords: Adenovirus; stem cell; gene therapy; regenerative medicine; review

Introduction

Adenovirus (Ad) is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm. At least 51 serotypes of human Ad have been identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) and Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, the penton base, and the fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Figure 1A). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: the tail, the shaft, and the knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber to the Ad capsid through association with the penton base.[1] The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation.[2] By extending the knob away from the virion, the shaft facilitates its interaction with the host

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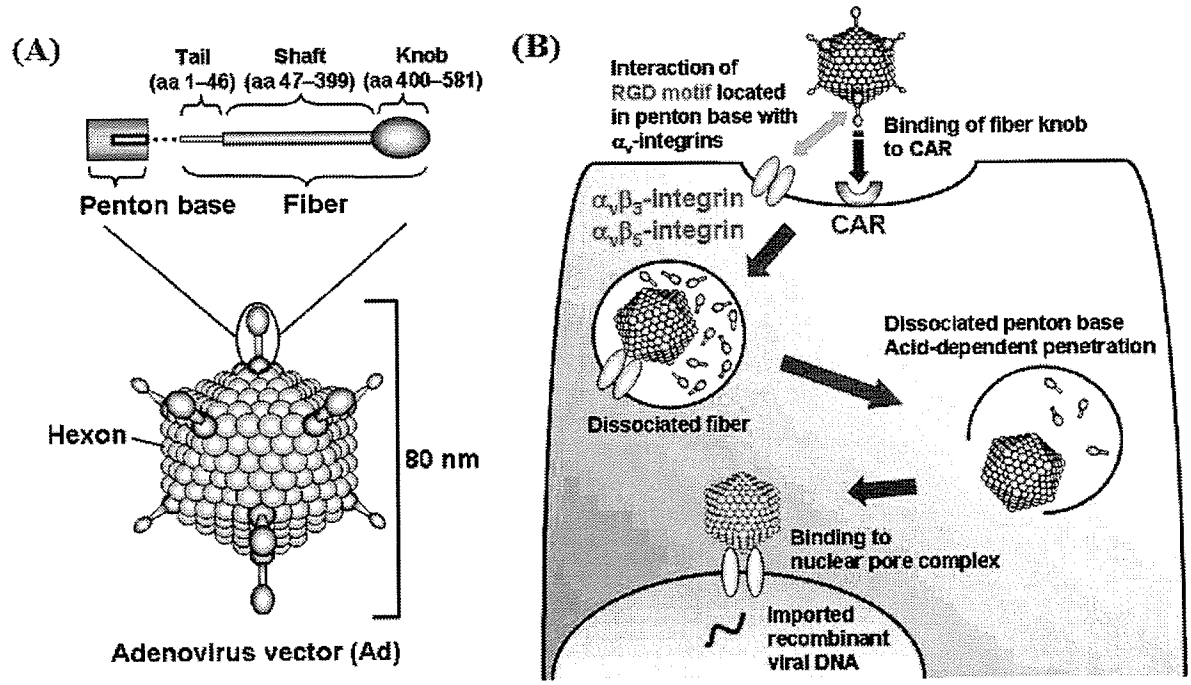

Figure 1. Structure and gene transduction pathway of the Ad vector. (A) The double-stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices. The fiber is composed of the tail, shaft, and knob domain. (B) The Ad vector binds to CAR following internalization in the cells and releases the viral DNA into the nuclei.

receptor.[1] The trimeric subunits of the carboxyl C-terminal knob domain are responsible for binding to the host's primary cellular receptor.[3,4]

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2), and five late units (L1−L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITRs) at the end of the viral genome function as replication origins. The E1A gene is the first transcription unit to be activated shortly after infection and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus propagated in E1-transcomplementing cell lines, such as 293,[5] 911,[6] or PER.C6 cells.[7] The E3 region-encoded proteins modulate the host defense but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted[8] and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer,[9] E1/E3-deleted Ad vectors allow the packaging of approximately 8.1−8.2 kb of foreign genes.[8]

The coxsackievirus and adenovirus receptor (CAR), which is a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A and C−F.[10−12] The entry of Ad5 into cells is initiated by the

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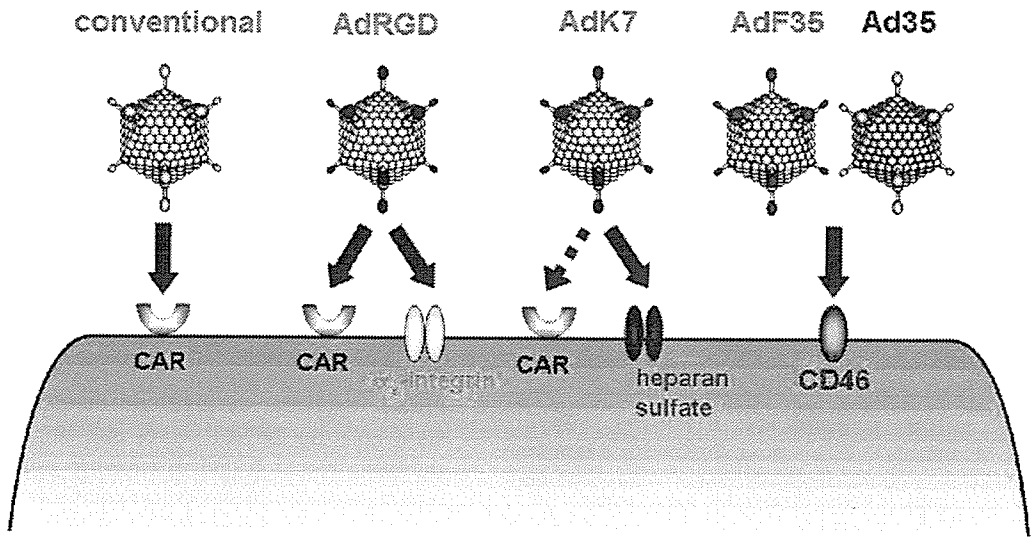

Figure 2. Characteristics of gene delivery by various types of Ad vectors. The conventional Ad vector infects via CAR. The AdRGD vector contains a RGD peptide motif in the HI loop of the fiber knob and infects via αv integrin as well as CAR. The AdK7 vector contains a polylysine peptide in the C-terminus of the fiber knob and infects via heparan sulfate as well as CAR. It is uncertain whether the AdK7 vector infects via CAR. The Ad35 and AdF35 vectors, which contain a fiber protein derived from the Ad5 fiber tail and the Ad35 fiber knob and shaft, infect via CD46.

attachment of fiber on the surface of the capsid to the CAR on the cell surface (Figure 2). The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid for the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$, aids in the internalization of Ad5 into the cell.[13−15] Furthermore, heparan sulfate glycosaminoglycans have also been reported to serve as primary attachment sites for Ad2 and Ad5.[16] The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation (Figure 1B). Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base.[17] The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus (Figure 1B). During this process, the terminal protein plays a crucial role in translocating the Ad genome into the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with the translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell.[18]

Although Ad vectors mediate extremely high transduction efficiency, gene transfer with Ad vectors is less efficient in some kinds of cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), dendritic cells, T cells, smooth muscle cells, skeletal muscle cells, and others because of the scarcity of CAR on their cell surfaces. Modification of the Ad fiber proteins has been used to successfully overcome this obstacle.[19,20] One is constructed by the addition of foreign peptides to the HI loop or C-terminus of the fiber knob of an Ad vector.[21−25] Enhanced gene transfer has been

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reported, on the basis of the use of mutant fiber proteins containing either an RGD peptide (AdRGD vector)[21−26] or a stretch of lysine residues [K7 (KKKKKKK) peptide] (AdK7 vector),[21,25,26] which target αv integrins or heparin sulfates on the cell surface, respectively (Figure 2). Altered vector tropism was reported with the substitution of the Ad5 fiber protein with that of Ad belonging to subgroup B, such as Ad types 3, 11, and 35.[27−31] These fiber-modified Ad vectors infect cells via CD46, CD80, and CD86, which have recently been identified as the cellular receptors of Ad belonging to subgroup B (Figure 2).[32−36] Mercier et al. described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing junctional adhesion molecule 1.[37]

Several groups have developed an Ad vector from the entire Ad type 35 (Ad35) or Ad type 11 (Ad11) and have demonstrated that the Ad35 and Ad11 vectors exhibit higher transduction efficiencies into hematopoietic progenitor and dendritic cells compared with the conventional Ad5 vector (Figure 2).[38−43] As other approaches to changing the vector tropism, modification of the Ad vector with the antibodies, the fusion protein composed of CAR and the cell binding domain, cationic lipid, or macromolecules has been reported.[19,20] Here, we highlight the genetic manipulations of stem cells by the Ad vector and fiber-modified Ad vector for basic research and therapeutic usage. Recent advances in Ad vector-mediated gene transfer into stem cells, such as embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), will be discussed.

Gene Transfer into Stem Cells

Stem cells are defined as cells which possess the abilities of self-renewal and multilineage differentiation. Stem cells have been isolated from a wide variety of tissues, and in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals can differentiate into specialized cells with a phenotype distinct from that of their precursor. Gene therapy applications that target stem

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cells offer great potential for the treatment of many kinds of diseases. Despite this promise, clinical success has been limited by poor rates of gene transfer and poor levels of gene expression. Therefore, an efficient gene delivery system needs to be developed for stem cell gene therapy.

Gene Transfer into Embryonic Stem Cells. ES cells are pluripotent cell lines derived from the inner cell mass of the developing blastocyst.[44−46] With the establishment of human ES (hES) cells, they have been used as a renewable source of transplantable tissue-specific stem cells.[47−49] ES cells differentiate spontaneously in vitro in a random manner into a mixture of differentiated cells. The protocols for the differentiation of ES cells enriched for a specific lineage have been developed in both the mouse ES (mES)[50,51] cell and hES cell systems,[52,53] although the differentiated cells are still relatively heterogeneous. Therefore, further research is needed to allow controlled directed differentiation of ES cells into pure cultures of committed cells. One of the most powerful techniques for controlled differentiation is genetic manipulation. Electroporation methods,[54] retroviral vectors,[55,56] lentiviral vectors,[57−59] and a supertransfection method based on a replication system using the polyoma replication origin and large T antigen[60] have been used for exogenous gene expression in ES cells, although lentiviral vectors have been shown to be ineffective at expressing exogenous genes in mES cells, but not in hES cells.[57,59] In plasmid-based systems such as eletroporation and supertransfection methods, stable cell lines are generated by selection using a drug resistance gene. All these methods mediate long-term constitutive gene expression, although a long-term gene expression system such as that as described above may be problematic for use in therapeutic applications, because the gene is continuously expressed even after cell differentiation. There is thus a need for efficient vector systems for transient expression.

The Ad vector has been thought to be inappropriate for gene transfer into ES cells.[61] It has been reported that the retrovirus vector preferentially transduced ES cells, while the Ad vector containing the cytomegalovirus (CMV) promoter preferentially transduced embryonic fibroblasts as feeders in the ES culture.[61] However, it was found that the

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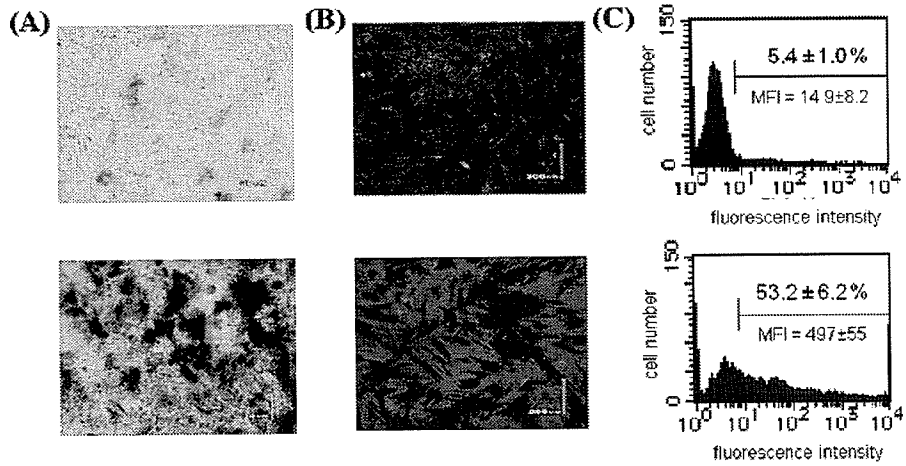

Figure 3. Improved transduction efficiency in the stem cells by the optimized Ad vectors. (A) mES cells were transduced with the LacZ-expressing conventional Ad5 vector containing the CMV promoter (top) or EF-1α promoter (bottom). (B) hMSCs were transduced with the LacZ-expressing Ad5 vector (top) or AdK7 vector (bottom). Both vectors have the CA promoter. (C) Human CD34+ cells were transduced with the GFP-expressing Ad5 vector (top) or Ad35 vector (bottom). Both vectors have the CMV promoter. MFI is the mean fluorescence intensity.

choice of a promoter is important for the efficient expression of exogenous genes in mES cells (Figure 3A). In the transient expression system using a cationic liposome−plasmid complex, the EF-1α (elongation factor 1α) and CA promoter (β-actin promoter/CMV enhancer) were shown to be highly active in mES cells while the CMV promoter was inactive.[62] More recently, we reported that the Ad vector containing the EF-1α or CA promoter has mediated the efficient expression of the reporter gene in mES cells, whereas the Ad vector containing the Rous sarcoma virus (RSV) or the CMV promoter has exhibited little expression.[63] Because CAR was highly expressed in mES cells but not in feeder cells,[63] the Ad vector could be a powerful tool for the genetic manipulation of mES cells when an appropriate promoter is used. To date, although we have no idea about the expression of CAR in hES cells, the Ad vector was reported to mediate the reporter gene expression in both mES cells and hES cells,[64] suggesting that hES cells may also express CAR on their cell surfaces.

As a result of the comparative analysis of mES cells transduced with various types of fiber-modified Ad vectors, the conventional Ad vector exhibited highly efficient and specific transduction, whereas the AdRGD and AdK7 vectors transduced mES cells and feeder cells (embryonic fibroblasts) to the same degree.[63] Therefore, the conventional Ad vector containing the EF-1α or CA promoter should be appropriate when only ES cells are transduced. In turn, the AdRGD or AdK7 vector is adequate when both ES cells and feeder cells are transduced.

The conventional Ad vector containing the EF-1α promoter was applied for the transduction of functional genes. It is well-known that the activation of signal transducer and activator of transcription 3 (STAT3) is essential for leukemia inhibitory factor (LIF)-mediated mES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation.[65] It is also known that transcription factor Nanog maintains the pluripotency of mES cells in a manner that is independent of LIF/STAT3 signaling.[66,67] Ad vector-mediated STAT3F (STAT3 dominant-negative mutant) transduction strongly promoted mES cells to cell differentiation into three germ layers without any nonspecific toxicity.[63] The co-infection of the STAT3F-expressing Ad vector and the Nanog-expressing Ad vector showed that the differentiation suppressing ability of Nanog negated the differentiation promoting function of STAT3F and that mES cells maintained their undifferentiated state.[63] Thus, the differentiation of ES cells could be controlled by the transduction of differentiation-key regulator genes with the Ad vector. ES cells might differentiate into hematopoietic progenitor, pancreatic β cells, or neurons by the Ad vector-mediated introduction of HoxB4,[68,69] Pax4,[70] or nuclear receptor-related I,[71] respectively.

Gene Transfer into Mesenchymal Stem Cells. MSCs, which reside within the stromal compartment of bone

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marrow, were first identified as bone-forming progenitor cells from rat marrow.[72] MSCs represent a very small fraction, 0.001−0.01% of the total population of nucleated cells in marrow.[73] They have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage, and muscle. Recently, it has been reported that MSCs can differentiate into other lineages, such as neurons,[74] hepatocytes,[75] and insulin-producing cells.[76] Therefore, MSCs have attracted a great deal of interest because of their potential use in regenerative medicine and tissue engineering. To date, MSCs could be differentiated in vitro into proper lineages via a change in the culture conditions.[77] Another method for the in vitro differentiation is to genetically modify MSCs.[78,79] Although exogenous gene transfer into human MSCs (hMSCs) has been reported by using a conventional Ad vector, its transduction efficiency is quite low due to the scarcity of CAR.[80,81] Therefore, hMSCs have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors.[80,81] Fiber-modified Ad vectors have been applied for hMSCs to improve the transduction efficiency.[79,82,83] hMSCs infected with the AdRGD vector containing the BMP2 gene produced larger amounts of BMP2 than cells infected with the conventional Ad vector and efficiently differentiated into the osteogenic lineage.[82,83] Highly efficient transduction of hMSCs was achieved with tropism-modified Ad5 vectors carrying fiber shaft domains and knobs of different serotypes of Ad, such as Ad16, Ad35, or Ad50.[84] In a systematic comparison with various types of fiber-modified Ad vectors, the AdK7 vector is the most efficient for hMSCs and exhibited a 460-fold higher transduction efficiency than the conventional Ad vector.[79] The AdRGD vector or the Ad vector containing the Ad35 fiber (AdF35) exhibits a 16 or 130 times higher transduction efficiency, respectively, than the conventional Ad vector.[79] hMSCs are found to express CD46, which is the primary receptor for Ad35, but not CAR.[79] In conclusion, the AdK7 or AdF35 vector is the most appropriate for the transduction of hMSCs (Figure 3B).

Gene Transfer into Hematopoietic Stem Cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and multilineage differentiation into all mature blood cells.[85] HSCs comprise only 0.01% of the whole bone marrow, the tissue in which they primarily reside.[86] Efficient transduction into HSCs would afford the opportunity to treat a number of hematopoietic disorders and would be a powerful tool for

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the study of the proliferation, differentiation, and trafficking of HSCs. Although the retroviral and lentiviral transduction of HSCs to achieve stable gene expression has been established,[87,88] stable expression is not always desirable. For example, stable expression of MDR1 gene results in HSC expansion but can cause leukemia upon transplantation to recipient mice.[89] As the Ad vector mediates the exogenous gene expression transiently, this vehicle can be safe for gene therapy. However, the application of conventional Ad vectors for the transduction into human CD34+ cells, which contain a population of HSCs, has been limited because CAR is not expressed at sufficient levels in human CD34+ cells.[90,91] It has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[90,92] We showed that the Ad35 vector, which is composed from the whole Ad35, achieved higher levels of transduction efficiency in human bone marrow CD34+ cells than both conventional Ad5 vectors and AdF35 vectors.[39,93] The expression level of reporter genes in the CD34+ cells transduced with the Ad35 vector was 12−76 and 1.4−3 times higher than that in the cells transduced with the Ad5 and AdF35 vectors, respectively.[39] The transduction efficiency of the Ad35 vector was slightly higher than that of the AdF35 vector, although the reason remains unknown. CD46 is ubiquitously expressed in almost all human cells, including human cord blood CD34+ cells.[94] Therefore, human CD34+ cells would be considered to be a suitable target for the Ad35 vector (Figure 3C). As a result of the systematic comparison of promoters with Ad35 vectors, significantly higher transduction efficiencies were achieved with the EF-1α, CA, and CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi) promoters. In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature subsets.[93] In mice, a population of mouse bone marrow highly enriched for HSC, called side population (SP) cells, has been reported to be transduced with the conventional Ad5 vector.[95] This suggests that pure mouse HSCs might express CAR on the cell surface. Further studies are needed to clarify this. The Ad vector-mediated transduction of hematopoietic regulator genes, such as HoxB4,[68,69] Bmi-1,[96] or SCL/Tal-1,[97] into HSCs may be effective for therapeutic use such as HSC expansion, although the Ad vector expressing HoxB4 was unsuccessful because of unexpected HSC differentiation due to its high transduction efficiency.[98]

Conclusions

We have reviewed recent advances in the development of improved Ad vectors for stem cells. Ad vectors have advantages over other viral vectors: the high transduction efficiency, the ease of vector preparation, and the transient expression ability. By the Ad vector-mediated introduction of a differentiation master regulator gene, we could control the differentiation of stem cells. These technical advances should greatly facilitate the analysis of gene function in the stem cells as well as the therapeutic applications of gene-modified stem cells.

Abbreviations Used

ES, embryonic stem; mES, mouse ES; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; Ad, adenovirus; CAR, coxsackievirus and adenovirus receptor; Ad5, Ad serotype 5; ITR, inverted terminal repeats; Ad35, Ad serotype 35; AdRGD vector, Ad vector containing the RGD

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peptide; Ad K7 vector, Ad vector containing a polylysine stretch; hES, human ES; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; STAT3F, dominant-negative mutant of STAT3; hMSCs, human MSCs; BMP2, bone morphogenetic protein 2; AdF35, Ad vector containing the Ad35 fiber.

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Approaches to improving the kinetics of adenovirus-delivered genes and gene products

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Abstract

Adenovirus (Ad) vectors have been expected to play a great role in gene therapy because of their extremely high transduction efficiency and wide tropism. However, due to the intrinsic deficiency of their immunogenic toxicities, Ad vectors are rapidly cleared from the host, transgene expression is transient, and readministration of the same serotype Ad vectors is problematic. As a result, Ad vectors are continually undergoing refinement to realize their potential for gene therapy application. Even after 1999, when a patient fatally succumbed to the toxicity associated with Ad vector administration at a University of Pennsylvania (U.S.) experimental clinic, enthusiasm of gene therapists for Ad vectors has not waned. With great efforts from various research groups, significant advances have been achieved through comprehensive approaches to improving the kinetics of Ad vector-delivered genes and gene products.

Keywords: Immunogenic toxicities; Biodistribution; Cationic liposome; PEGylation; Helper-dependent; Targeting; In-cis acting element; Integration; Regulatable expression

Contents

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1. Introduction

Viral vectors show great promise for gene delivery in both basic research and therapeutic applications. It is vital to select the most appropriate viral vector for each specific application, and a number of factors must be taken into consideration when making such a selection. These include the efficiency and specificity with which the vector infects the target cells, the transgene size, the level and duration of the transgene expression, the question of whether regulation of the transgene is needed, and the level of toxicity that can be tolerated. There are now more than 10 viral vector types in use, derived from common human or mammalian viral pathogens including retrovirus, adenovirus, adeno-associated virus, lentivirus, herpes simplex virus, and poxvirus. However, there is no single viral vector type meeting all the requirements, and the methods for using viral vectors to deliver genes are continually being refined.

Adenoviruses (Ad) are nonenveloped viruses containing an icosahedral protein capsid with a diameter of approximately 80 nm. There are at least 51 serotypes of human Ad identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) along with Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, penton base, and fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Fig. 1). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: tail, shaft, and knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber

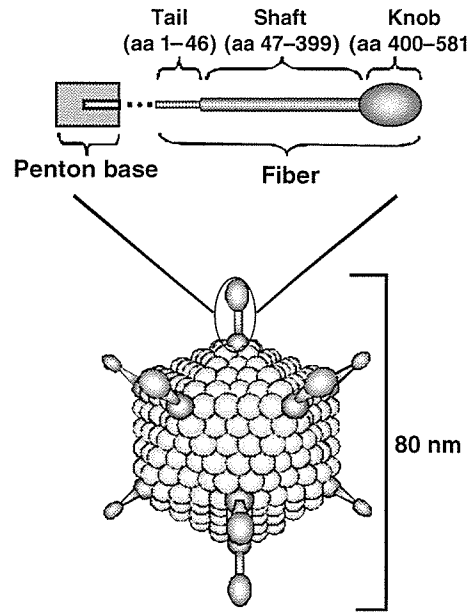

Fig. 1. Schematic diagram of a human Ad serotype 5 virion. The double stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices.

to the Ad capsid through association with the penton base [1]. The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation [2]. By extending the knob away from the virion, the shaft facilitates its interaction with host receptor [1]. The trimeric subunits of the carboxyl (C)-terminal knob domain are responsible for binding to the host's primary cellular receptor [3,4].

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding over 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two early delayed (intermediate) transcription units (pIX and IVa2) and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITR) at the end of the viral genome function as replication origins (Fig. 2). The E1A gene is the first transcription unit to be activated shortly after infection, and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus is propagated in E1-transcomplementing cell lines, such as 293 [5], 911 [6], or PER.C6 cells [7]. The E3 region-encoded proteins modulate the host defense, but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted [8], and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer [9], E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes [8].

The coxsackievirus and adenovirus receptor (CAR), a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A, C, D, E and F [10–12]. Entry of Ad5 into cells is initiated by the attachment of fiber on the surface of the capsid to the CAR on the cell surface. The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid to the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$, helps mediate the internalization of Ad5 into the cell [13–15]. Furthermore, heparan sulfate glycosamino-glycans have also been reported to serve as primary attachment sites for Ad2 and Ad5 [16]. The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base [17]. The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell [18].

Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and non-dividing cells. They have large packaging ability for foreign genes and can be easily grown to high titers and purified for clinical applications. Furthermore, Ad is nononcogenic, and Ad-related pathology is mostly limited to mild upper respiratory tract infections. All these advantageous features lead to increasing number

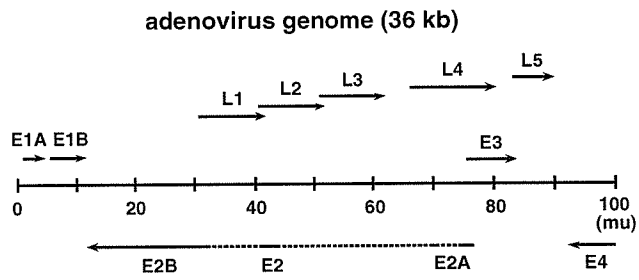

Fig. 2. Genome structure of human Ad serotype 5. The early transcription units E1 (and E3) are deleted for the first-generation Ad vectors.

of clinical protocols employing Ad vectors. As of October of 2003, the percentage of gene therapy protocols utilizing Ad vectors was at 27% (636 protocols) and the percentage of patients treated with Ad vectors at 18% (3496) (Journal of Gene Medicine Website, www.wiley.co.uk/genmed/clinical). This proportion is second only to retroviral vectors.

However, the immune response to the Ad vector-transduced cells dramatically affects the kinetics of Ad vector-delivered genes and the gene products. The potent immunogenic toxicities and consequent short-lived transgene expression of Ad vectors are undesirable properties if Ad vectors are to be more broadly applied. Though Ad vectors find niches in the treatment of degenerative diseases like vascular and coronary artery diseases in which transient transgene expression is advantageous [19,20], and for cancer, in which cellular toxicity and immunogenicity might enhance antitumor effects [21], less antigenic Ad vectors with long-term transgene expression are preferable in most cases. Here, we would like to highlight various approaches to overcoming the hurdle of Ad vector immunogenic toxicities to improve the kinetics of Ad vector-delivered genes and gene products.

2. Immunogenic toxicities of Ad vectors

The greatest obstacle for gene therapists wanting to exploit Ad vectors is the issue of their viral immunogenic toxicities. With the extensive efforts of various research groups, more and more data concerning Ad vector immunogenic toxicities have been obtained. However, it is apparent that the immunogenic toxicities associated with the use of Ad vectors are extremely complex, involving both innate and adaptive immune responses, along with apoptosis, and we are still far from a thorough understanding of all the aspects of the toxic interaction between Ad vectors and their hosts.

The complexity of Ad vector immunogenic toxicities lie in the fact that they induce multiple components of the immune response [22–24]. The cytotoxic T-lymphocyte (CTL) response can be elicited against viral gene products and/or transgene products expressed by transduced cells, resulting in a host immune attack against the transduced cells and the elimination of transgene-expressing cells. The Ad capsid itself can induce humoral virus-neutralizing antibody responses, which prevent transgene expression on subsequent administration of vectors of the same serotype, and which also provoke potent cytokine-mediated inflammatory responses during which NF κB activation might play a central role [25,26]. It is thought that among the inflammatory cytokines, TNF-α plays a dominant role in Ad vector clearance [26,27]. Wilson's group demonstrated that systemically administered Ad vectors preferentially activated dentritic cells and macrophages in the spleen to release inflammatory cytokines, independent of transgene expression [28,29]. This suggests in designing targeted Ad vectors, it should be taken into consideration to reduce transduction of or sequestration by antigen presenting cells.

Like all drug-associated toxicities, the degree to which Ad vectors induce harmful immune-mediated and inflammatory responses and other toxic side effects is dose-dependent [30–32]. Highly dangerous inflammatory responses might be inappropriately activated, especially at high doses of Ad vectors. During the tragic 1999 gene therapy trial for deficiency of ornithine transcarbamylase (OTC) at the University of Pennsylvania (U.S.), an escalated dose of Ad vector (3.8×10^{13} particles) systemically administered through the hepatic artery induced a massive systemic inflammatory response that led to fever, disseminated intravascular coagulation, multi-organ failure and the eventual death of an 18-year-old patient [33,34]. Besides the potential of a lethal outcome, immunogenic toxicities attenuate the therapeutic efficacy by affecting the kinetics of delivered genes and gene products.

3. Kinetics of Ad vector-delivered gene and gene product

It is known that different viruses are cleared from the blood stream by Kupffer cells (KC) [35]. However, blood clearance varies among different viruses [35]. Important determinants of virus clearance from the blood stream include interaction between viral components and cellular receptors, and virion size. The net charge of the viral particle also affects the clearance kinetics [36]. Interventions that slow the

clearance of Ad vectors from the blood stream might favor tissue- or tumor-specific targeting approaches based on systemic delivery [37,38]. Understanding the kinetics of blood clearance and biodistribution of Ad vectors would be beneficial to the advance of their application as therapeutic agents.

Several groups have verified that KC played a central role in clearing the Ad particles from the blood stream by blocking the KC of mouse liver [26,39,40]. The Curiel group's as well as our own, indicated rapid clearance of Ad vectors from the blood of mice, with a half-life of less than 3 min and negligible levels of the Ad vectors remaining in the blood 30 min after injection (Fig. 3A) [38,41,42]. To examine the role of the liver in the blood clearance of Ad vectors, we determined the amounts of Ad vector DNA in the mouse. In accordance with the other groups' data [40,43], we found that 98% of the intravenous dose had accumulated in the mouse liver 1 h after injection. Forty-eight hours after injection, 43% of the input Ad5 DNA persisted in the liver (Fig. 3B) [41]. These data confirm that, following intravenous administration, Ad vectors are predominantly delivered to the liver. Besides the strong interaction between viral components and cellular receptors (e.g. the fiber-CAR and RGD motif of the penton base-αv integrins), the inclination of Ad vectors to the mouse liver may also be attributed to the anatomical properties of the liver sinasoid [43,44]. The accumulation of Ad vectors in the liver may itself be toxic. We further investigated the cellular distribution of Ad vector genomes in mouse livers after intravenous injection, and found them to be equally distributed in the parenchymal cells (PC; hepatocytes) and nonparenchymal cells (NPC; Kupffer cells and endothelial cells), when 1.5×10^{10} particle of Ad vectors were intravenously injected into the mouse [41]. Despite the high uptake of Ad vectors by the NPC, the Ad vector-mediated transduction efficiencies in the NPC were much lower than those in the PC, indicating the uptake of Ad vectors by the NPC is a function of phagacytosis rather than a receptor-mediated infectious pathway [41]. This result of the high uptake of Ad vectors by the NPC combined with low transduction efficiencies is consistent with previous reports of nonlinear dose responses of Ad transduction in the liver [45,46]. Those results suggested that there was a viral dose threshold effect for efficient liver transduction of Ad vectors and that NPC played a central role in this threshold effect, such that low doses of Ad vectors were efficiently taken up by the NPC without appreciable transgene expression, while high doses saturated the NPC and were able to productively transduce the PC. Hence, depleting the Kupffer cells or blocking their uptake before Ad vector administration might be helpful in reducing the Ad vector dose for systemic route gene therapy [45,46].

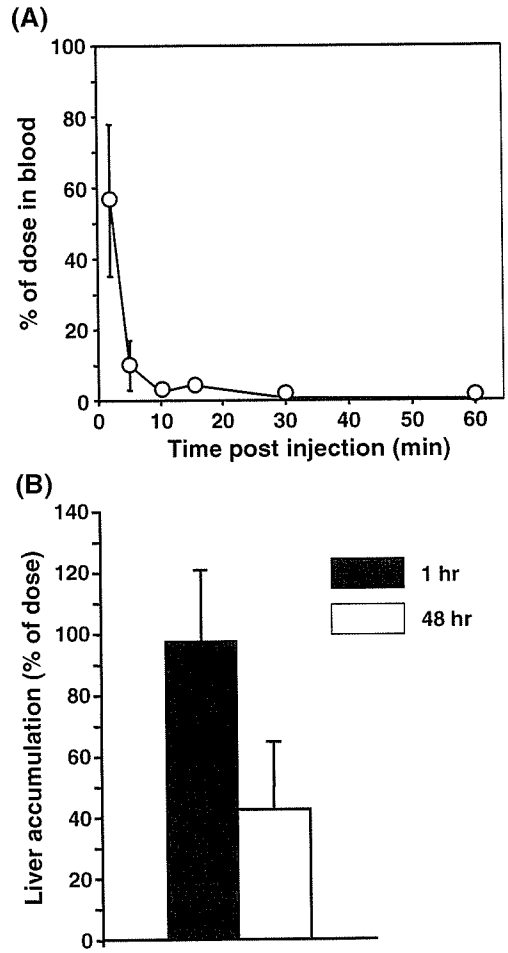

Fig. 3. Blood clearance kinetics and liver accumulation of Ad vectors after intravenous administration into mice. E1- and E3-deleted Ad vectors (1.5×10^{10} particle) were intravenously injected: (A) blood was drawn from the retro-orbital at the indicated times postinjection. (B) The livers were isolated 1 or 48 h after injection. Total DNA, including the Ad vector genome, was isolated from the blood or the livers, and slot-blot analysis was then performed. The data in this figure were published in our previous paper [41].

It is putative that transgene expression from Ad vectors is transient because of the rapid clearance of viral particles by the host immune response. However, duration of transgene expression may vary according to transgene products or animal species/strains. For example, marked variability was observed in the persistence of human alpha 1-antitrypsin (hAAT) expression delivered by an E1/E3-deleted Ad vector in different mouse strains, ranging from several weeks in the strains of C3H/HeJ and Balb/c to more than 3 months in the strains of C57Bl/6, B10.A(2R) and B10.BR [47]. This is because immunogenicity varies according to different transgene proteins, and immune responses (cellular and/or humoral) to invading virions vary according to different species or strains [48].

Overall, the kinetic features of Ad vector-delivered genes and gene products might be summarized as rapid clearance of virus from blood, liver accumulation of virus DNA and expression, and transient transgene expression. Moreover, unlike common-sense pharmaceuticals, readministration of Ad vectors is problematic due to neutralizing antibodies.

4. Approaches to improving the kinetics of Ad-delivered genes and gene products

The disadvantageous kinetics of Ad vector-delivered genes and gene products for gene therapy applications results from their immunogenic toxicities. All the potential approaches to improving the kinetics should be based on attenuating the immunologic interaction between Ad vectors and hosts, so as to extend the persistence of the virus in the blood, reduce the accumulation of the virus and transgene expression in the liver, prolong circulational or local transgene expression in the organ/tissue of interest, and make readministration possible.

4.1. Viral genome deletion

Ad vectors have been extensively engineered to reduce their immunogenicity. First-generation Ad vectors were deleted for only one or two viral early genes (E1 and E3). Cells transduced with these vectors expressed other Ad genes at low levels, inducing strong cytotoxic T-cell responses that rapidly eliminated transgene expression. Second-generation vectors that contain additional deletions in other early genes (E2 and/or E4) have shown reduced toxicity profiles compared to first-generation Ad vectors due to the decreased Ad protein synthesis in transduced cells [49–51]. However, the remaining viral gene expression still induces the T-cell response, which is difficult to overcome.

Progress has been made in reducing T-cell responses against viral gene products expressed by transduced cells, by engineering "helper-dependent" (HD) or "gutless" or "gutted" Ad vectors, from which all viral genes are deleted except the inverted terminal repeats (ITR) sequences at the two ends and the packaging signal of the Ad genome. The HD Ad vectors are produced with a helper Ad that provides in-trans the necessary viral proteins required for replication and packaging of the HD vector (Fig. 4). This advance has improved the prospects of Ad vectors for long-term gene transfer [52]. Several application experiments have shown that the HD Ad vectors have facilitated life-long phenotypic correction in mouse models with negligible toxicity. For example, in a mouse model of hyperlipidemia, a defect correction was observed for 2.5 years with a single injection of an HD Ad vector [53]. In another mouse model of hemophilia, expression of human factor VIII was sustained for longer than 9 months [54]. However, in canine models of hemophilia A or B, only transient phenotypic correction, and in some of those cases only partial correction, was observed with no detectable toxicity using an HD Ad vector [55,56], while the same group of researchers achieved long-term phenotypic correction in a mouse hemophilia B model without toxicity by a single injection of an HD Ad vector [57]. Though the discrepancy of transgene persistence by HD Ad vectors between mouse and large animal models still needs to be elucidated, it is clear that immunogenic toxicities induced by HD Ad vectors are greatly reduced.

Compared with early generation Ad vectors, yields of HD Ad vector production need to be increased, and caution should be taken to decontaminate helper Ad. Some advances have been achieved in these aspects. Sakhuja et al. [58] developed an optimized HD Ad vector production system by generating a novel producer cell line, PERC6-Cre, which was adapted to serum-free suspension culture for bioreactor mass

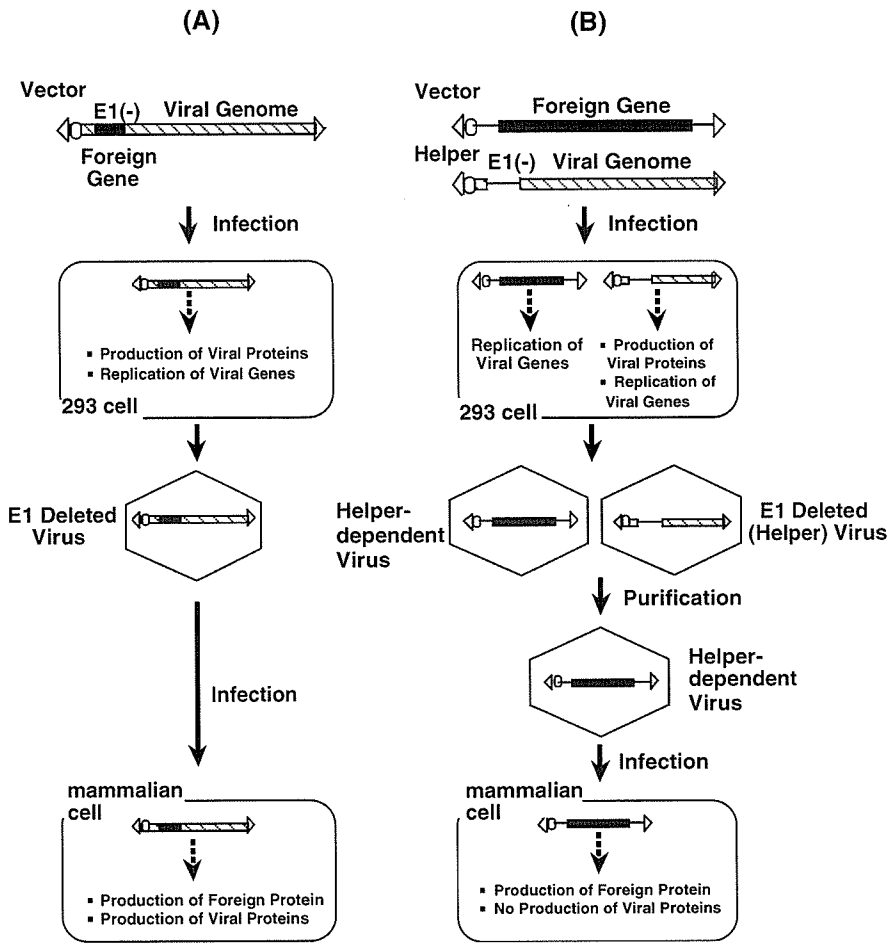

Fig. 4. Propagation diagrams of the first-generation Ad and helper-dependent Ad vectors. (A) The first-generation Ad vectors are produced in E1-transcomplementing cell lines, such as 293, 911, or PER.C6 cells. (B) Helper-dependent (HD) Ad viruses are propagated with the viral proteins provided by helper Ad viruses. To reduce the production of the helper virus, a Cre/loxP recombination system is generally utilized to excise the packaging signal from the helper virus genome [59].

production of HD Ad vectors. However, they also indicated that using the existing Cre/loxP technology to excise the packaging signal from the helper virus genome, which was originally developed by Graham and colleagues [59], could not completely eliminate the helper virus from HD Ad preparations.

With the advantages of reduced toxicity, larger packaging capacity for foreign genes of up to 36 kb, and possible persistent transgene expression, HD Ad vectors remain a powerful tool for gene therapy, though the humoral response against incoming capsid proteins shortly after administration remains a major challenge.

4.2. Modulation of the viral tropism

The broad tropism of Ad, on one hand, leads to unwanted vector uptake by many different cell types in multiple organs when the vectors are delivered systemically. Even the local delivery of Ad vectors can lead to leakage and dissemination to other tissue, resulting in toxic effects on distal sites, most notably the liver [60–62]. On the other hand, important types of target tissues are refractory to Ad infection due to CAR scarcity; these include primary tumor cells [63–66], mature skeletal muscle [67], endothelial [68,69], smooth muscle [68,69], differentiated airway epithe-