

Fig. 1 Schematic diagram of transduction mechanisms of adenovirus serotype 5 vectors and adenovirus serotype 35 vectors.

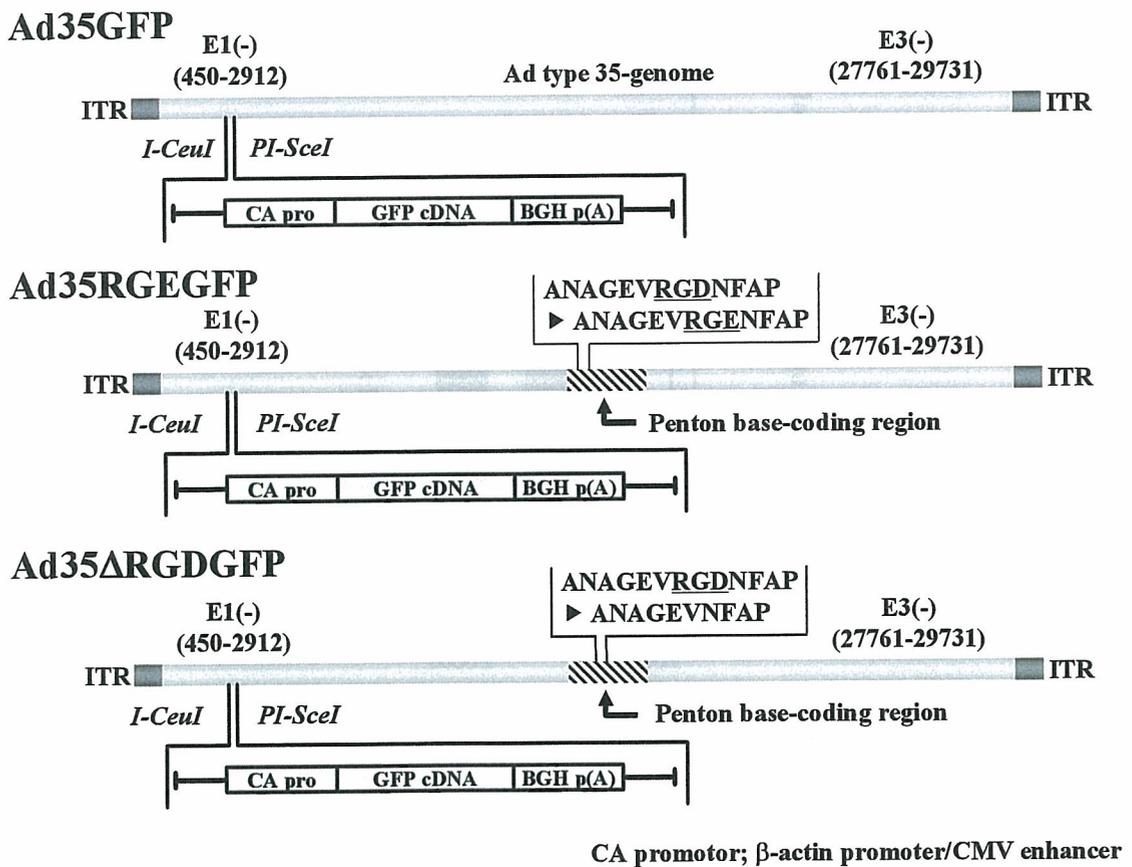


Fig. 2 Schematic representation of adenovirus used in this study

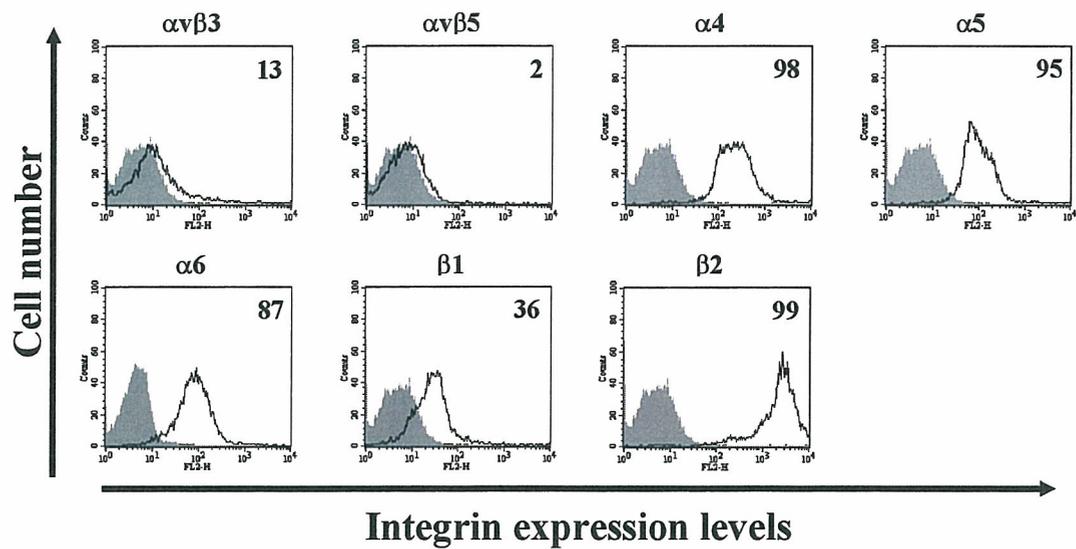


Fig. 3 Flow cytometric profiles of integrin expression on human bone marrow-derived CD34⁺ cells. The cells were stained with anti-integrin antibodies, followed by a PE-labeled secondary antibody, and subsequently analyzed by a flow cytometer (thick line). As a negative control, the cells were incubated with isotype control antibody (shaded histogram). Percentage of positive cells is shown by number in upper right-hand corner of each profile.

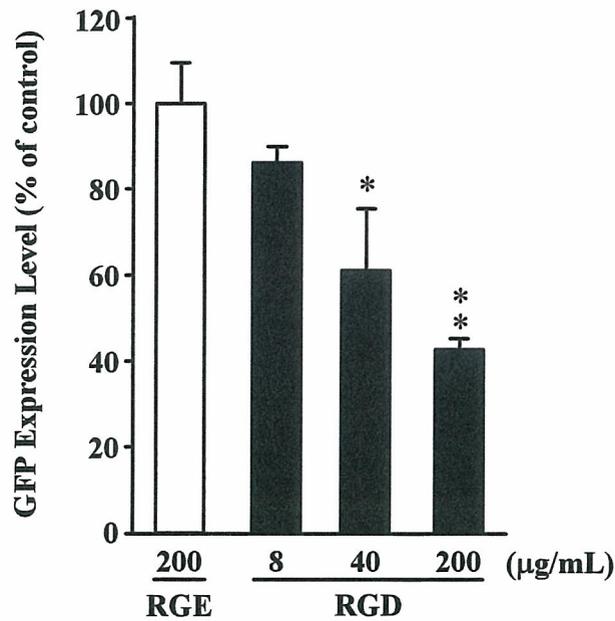


Fig. 4 Inhibition of Ad35 vector-mediated transduction by RGD synthetic peptides. Human CD34⁺ cells were incubated with RGD synthetic peptides at the indicated concentrations at 4 °C for 1 h prior to the addition of Ad35GFP, and then left for 3 h at 37 °C. Samples were then washed, resuspended in a fresh medium, and incubated at 37 °C. GFP expression levels were measured 48 h after transduction by flow cytometry. The data were normalized to the GFP expression levels (MFI) in human CD34⁺ cells in the presence of control RGE peptides. The data are expressed as the mean \pm S.D. ($n=3$) * $P < 0.05$, ** $P < 0.001$ for comparison with the cells pretreated with control RGE peptides.

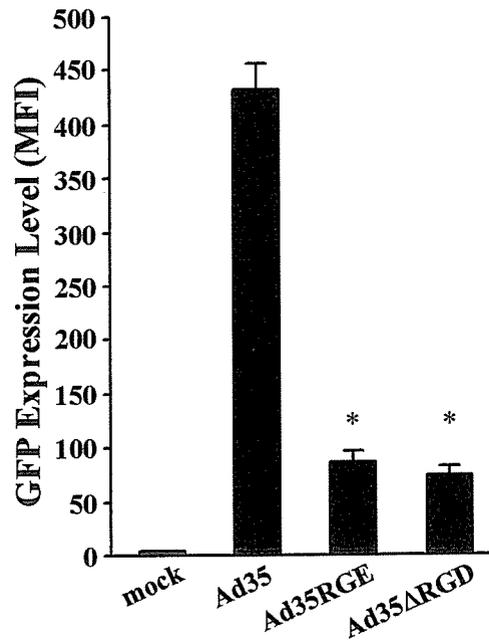


Fig. 5 Comparison of GFP expression levels in human CD34⁺ cells transduced with Ad35GFP, Ad35RGE, and Ad35ΔRGD. Human CD34⁺ cells were transduced with 6000 VP/cell of Ad35GFP, Ad35RGE, and Ad35ΔRGD for 6 h at 37 °C. GFP expression levels (MFI) were measured 48 h after transduction using flow cytometry. The data are expressed as the mean \pm S.D. ($n=3$) * $P < 0.001$ for comparison with the cells transduced with Ad35GFP.

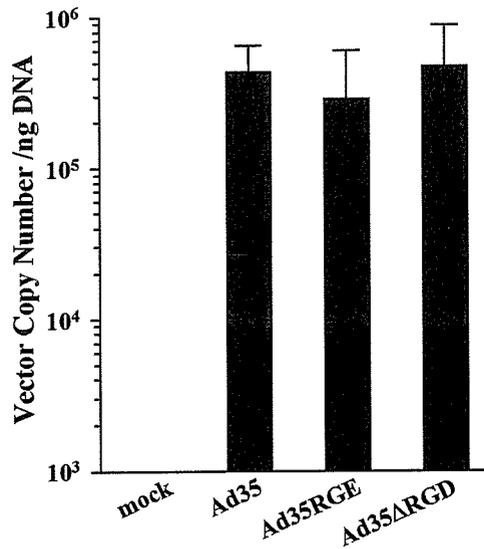


Fig. 6 Viral uptake of several Ad35 vectors in human CD34⁺ cells. The cells were transduced with Ad35GFP, Ad35RGE, and Ad35ΔRGD for 3 h at 37 °C. After incubation, the cells were washed five times with ice-cold PBS, and total DNA, including the Ad vector DNA, was extracted. The copy numbers of the Ad vector DNA were quantified by TaqMan PCR. The data are expressed as the mean \pm S.D. ($n=4$)

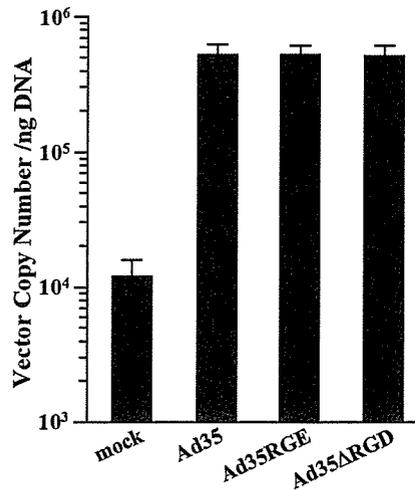


Fig. 7 Cellular attachment of Ad35 vectors on human CD34⁺ cells. Human CD34⁺ cells were incubated with Ad35GFP, Ad35RGEGFP, and Ad35ΔRGDGFP for 3 h at 4 °C. After incubation, the cells were washed five times with ice-cold PBS, and total DNA, including the Ad vector DNA, was extracted. The copy numbers of the Ad vector DNA were quantified by TaqMan PCR. The data are expressed as the mean \pm S.D. ($n=4$)

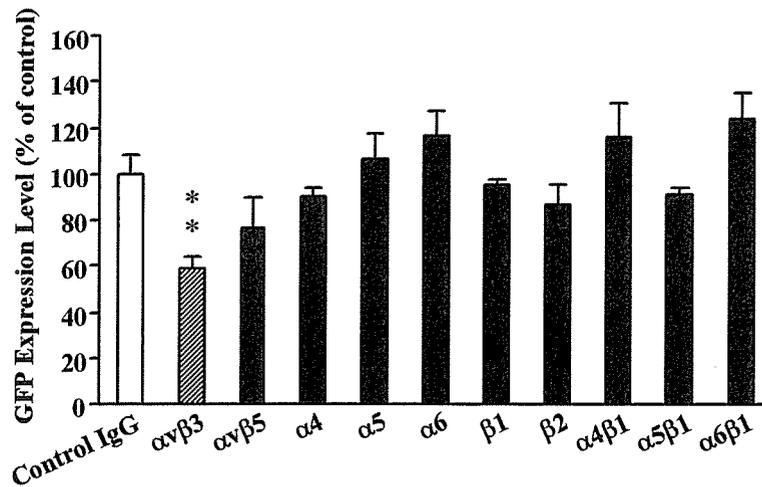


Fig. 8 Inhibition of Ad35 vector-mediated transduction by monoclonal anti-integrin antibodies. Human CD34⁺ cells were preincubated with each antibody at 50 μg/mL for 1 h at 4 °C, and then added 3000 VP/cells of Ad35GFP. The cells were incubated at 3 h at 37 °C, and then cells were washed, and resuspended in a fresh medium and incubated at 37 °C. GFP expression levels were measured 48 h after transduction using flow cytometry. The data were normalized to the GFP expression levels (MFI) in human CD34⁺ cells in the presence of control mouse IgG. The data are expressed as the mean \pm S.D. ($n=3$) * $P < 0.05$, ** $P < 0.01$ for comparison with the cells preincubated with control IgG.

研究成果の刊行に関する一覧表

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Technical Report

Rapid Construction of Small Interfering RNA-Expressing Adenoviral Vectors on the Basis of Direct Cloning of Short Hairpin RNA-Coding DNAs

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ABSTRACT

In the conventional method for constructing an adenoviral (Ad) vector expressing small interfering RNA (siRNA), short hairpin RNA (shRNA)-coding oligonucleotides are introduced downstream of a polymerase III (or polymerase II)-based promoter cloned into a shuttle plasmid. An siRNA expression cassette, which is cloned into the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid by *in vitro* ligation or homologous recombination in *Escherichia coli*, and the linearized plasmid is transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step plasmid manipulation is required. In this study, we developed a method by which shRNA-coding oligonucleotides can be introduced directly into the Ad vector plasmid. To do this, we constructed a new vector plasmid into which the human U6 promoter sequence was cloned in advance. Unique restriction enzyme sites were introduced at the transcription start site of the U6 promoter sequence in the vector plasmid. Luciferase and p53 genes were efficiently knocked down by Ad vectors generated by the new method and expressing siRNA against the target gene. This method should be useful for RNA interference-based experiments, and should make it easy to construct an siRNA-expressing Ad vector library for functional screening.

INTRODUCTION

RNA INTERFERENCE (RNAi), which mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologous to the target gene (Scherer and Rossi, 2003), is a powerful tool for the knockdown of gene expression. Transduction of synthetic small interfering RNA (siRNA; 19 to 29 nucleotides of RNA) or the promoter-based expression of siRNA in the cells results in sequence-dependent degradation of target mRNA and subsequent reduction of target gene expression. Most promoter-based RNAi systems express short hairpin RNA (shRNA), which is then trimmed by Dicer, generating functional siRNA. Polymerase III-based promoters, such as the small nuclear RNA U6 pro-

motor or the human RNase P RNA H1 promoter, are widely used for the expression of shRNA (siRNA) (Scherer and Rossi, 2003), although polymerase II-based promoters are also used (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). The promoter-based method has an advantage in that viral vectors as well as nonviral vectors can be used for delivery of the siRNA expression unit, whereas only nonviral vectors are used for delivery of synthetic siRNA.

Recombinant adenoviral (Ad) vectors have been used extensively to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo* (McConnell and Imperiale, 2004; Volpers and Kochanek, 2004). They can be easily grown to high titers and can efficiently transfer genes into both dividing and nondividing cells. Therefore, Ad vector-mediated

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delivery of an siRNA expression unit, in which a promoter-based shRNA expression cassette is delivered into the cell by the Ad vector, provides a valuable tool for both gene function studies and therapeutic applications.

Construction of Ad vectors used to be a time-consuming and labor-intensive procedure, but several improved methods to facilitate the construction of Ad vectors have been developed (reviewed in Mizuguchi *et al.*, 2001). The homologous recombination method in E1-complementing cell lines (i.e., 293 cells) has been the most widely used method for generating recombinant Ad vectors, and it has greatly contributed to the widespread use of Ad vectors (Bett *et al.*, 1994). The major limitations of this approach are the low frequency of the recombination event and the tedious and time-consuming plaque purification procedure required to select the recombinant virus of interest, because a relatively high percentage of the virus produced is wild type (in most cases, 20–70%), due to recombination with the Ad sequence integrated into the chromosomes of 293 cells. The improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999) and the homologous recombination method in *Escherichia coli* (He *et al.*, 1998), which are commercially available from Clontech (Palo Alto, CA) and Invitrogen (Carlsbad, CA), respectively, have now become widely used, because these systems overcome the limitations of the homologous recombination method in 293 cells. To construct an Ad vector expressing siRNA by these two methods, shRNA-coding oligonucleotides are introduced downstream of the polymerase III (or polymerase II)-based promoter cloned in a shuttle plasmid. An shRNA (siRNA) expression cassette, which is cloned in the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid, which clones a full Ad genome, by simple *in vitro* ligation or homologous recombination in *E. coli*. The resulting plasmid is then linearized and transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step *E. coli* transformation and plasmid manipulation is required for the improved *in vitro* ligation method, whereas three-step *E. coli* transformation and plasmid manipulation is required in the homologous recombination method in *E. coli* (because a special *E. coli* strain is used in the latter method, retransformation into a normal strain of *E. coli* is required) (reviewed in Mizuguchi *et al.*, 2001).

In the present study, we developed a simple method for generating an Ad vector expressing siRNA, in which shRNA-coding oligonucleotides could be directly introduced into an Ad vector plasmid containing the human U6 (hU6) promoter sequence. Unique restriction enzyme sites were introduced at the transcription start site of the hU6 promoter sequence cloned into the Ad vector plasmid. Two types of modified hU6 promoter sequence were constructed to develop this method. Using this method, only one-step *E. coli* transformation is required to generate an Ad vector plasmid containing an siRNA expression cassette.

MATERIALS AND METHODS

Cells

A549 and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf

serum (FCS). A549-Luc cells, which are stable transformants with luciferase expression, were cultured in DMEM supplemented with 10% FCS. For construction of A549-Luc cells, A549 cells were transfected with luciferase-expressing plasmid pGL3-Control-RSVneo, which contains the simian virus 40 (SV40) promoter/enhancer-luciferase cDNA-SV40 p(A) sequence and the neomycin expression cassette, using SuperFect transfection reagent (Qiagen, Valencia, CA). pGL3-Control-RSVneo was constructed by insertion of the Rous sarcoma virus (RSV) promoter-driven neomycin expression cassette into pGL3-Control (Promega, Madison, WI). Monoclonal A549 cells stably expressing luciferase (A549-Luc) were obtained by geneticin (G418) selection.

Plasmid and virus

The hU6 promoter sequence was amplified from human genomic DNA (Clontech), using the following primers: hU6-S1, hU6-AS1, and hU6-AS2 (Table 1). The hU6a and hU6b promoter sequences were amplified with hU6-S1/hU6-AS1 and hU6-S1/hU6-AS2 primer sets, respectively (see Fig. 2). These promoter sequences were introduced into pHM5 (Mizuguchi and Kay, 1999), and were then transferred into the E1 deletion region of the vector plasmid pAdHM4.1, a derivative of pAdHM4 (Mizuguchi and Kay, 1998) (the *Xba*I site outside the Ad genome of pAdHM4 was deleted), by an *in vitro* ligation method using the *I-Ceu*I and *PI-Sce*I sites (Mizuguchi and Kay, 1998, 1999), resulting in pAdHM4-hU6a and pAdHM4-hU6b, respectively (Fig. 1A). To construct a vector plasmid containing an shRNA-coding sequence against luciferase, oligonucleotides 1/2 and 3/4 were synthesized (Table 1), annealed, and cloned into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-Lu and pAdHM4-hU6b-Lu, respectively. The target sequence for siRNA is bp 158 to 176 of luciferase cDNA. For the construction of vector plasmid containing shRNA-coding sequence against p53 (Brummelkamp *et al.*, 2002), oligonucleotides 5/6 and 7/8 were used for cloning into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-p53 and pAdHM4-hU6b-p53, respectively. The target sequence for siRNA is bp 775 to 793 of human p53 cDNA.

The original intact hU6 promoter sequence, derived from an *Eco*RI/*Sa*II fragment of piGene hU6 (iGENE Therapeutics, Tsukuba, Japan), was also introduced into the *Sph*I and *Sa*II sites of pHM5 (Mizuguchi and Kay, 1999), resulting in pHM5-ihU6. pHM5-ihU6 was then digested with *Sa*II and *Xba*I, and ligated with oligonucleotides 9 and 10, resulting in pHM5-hU6. In this case, oligonucleotides 11/12 and 13/14 (for the shRNA-coding sequence against luciferase and p53, respectively) were introduced into the *Bsp*MI site of pHM5-hU6 according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); and then an siRNA expression cassette was inserted into the E1-deletion region of pAdHM4 (Mizuguchi and Kay, 1998), using the *I-Ceu*I and *PI-Sce*I sites, resulting in pAdHM4-hU6-Lu and pAdHM4-hU6-p53, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Viruses (Ad-hU6-Lu, Ad-hU6a-Lu, Ad-hU6b-Lu, Ad-hU6-p53, Ad-hU6a-p53, and Ad-hU6b-p53) were prepared by the

TABLE 1. OLIGONUCLEOTIDES USED IN THE PRESENT STUDY

Oligonucleotide	Sequence of oligonucleotide (5'–3')
hU6-S1 primer	aaggtcgggcaggaagaggccta
hU6-AS1 primer	<u>ggtctagaagtagt</u> <u>cgatttcgctttccacaagatat</u> (<i>Xba</i> I and <i>Cl</i> aI recognition sequences are underlined and italicized, respectively)
hU6-AS2 primer	<u>ggtctagaagtagt</u> <u>atataa</u> <u>tcgctttccacaagatatataa</u> (<i>Xba</i> I and <i>Swa</i> I recognition sequences are underlined and italicized, respectively)
Oligonucleotide 1	<u>cgacgctgagtagt</u> <u>ctcga</u> <u>aaattcaagaga</u> <u>atttcgaagtagt</u> <u>cagcgtttttggaat</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 2	<u>ctagattccaaaaaacgctgagtagt</u> <u>ctcga</u> <u>aaatttcga</u> <u>atttcgaagtagt</u> <u>cagcgt</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 3	<u>ccacgctgagtagt</u> <u>ctcga</u> <u>aaattcaagaga</u> <u>atttcgaagtagt</u> <u>cagcgtttttggaat</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 4	<u>ctagattccaaaaaacgctgagtagt</u> <u>ctcga</u> <u>aaatttcga</u> <u>atttcgaagtagt</u> <u>cagcgtgg</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 5	<u>cgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtc</u> <u>ttttggaat</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 6	<u>ctagattccaaaaaacgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtc</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 7	<u>ccgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtc</u> <u>ttttggaat</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 8	<u>ctagattccaaaaaacgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtcgg</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 9	<u>tcgacctgcagc</u> <u>atgcaagcttc</u> (<i>Bsp</i> MI recognition sequences are underlined)
Oligonucleotide 10	<u>ctaggaagcttgc</u> <u>atgcaagcttc</u> (<i>Bsp</i> MI recognition sequences are underlined)
Oligonucleotide 11	<u>caccacgctgagtagt</u> <u>ctcga</u> <u>aaattcaagaga</u> <u>atttcgaagtagt</u> <u>cagcgttttt</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 12	<u>gcataaaaaacgctgagtagt</u> <u>ctcga</u> <u>aaatttcga</u> <u>atttcgaagtagt</u> <u>cagcgt</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 13	<u>caccgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtc</u> <u>ttttt</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 14	<u>gcataaaaaaacgactccagtggaat</u> <u>tactctcaagaga</u> <u>tagattaccactggagtc</u> (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)

transfection of a *Pac*I-linearized vector plasmid (pAdHM4-hU6-Lu, pAdHM4-hU6a-Lu, pAdHM4-hU6b-Lu, pAdHM4-hU6-p53, pAdHM4-hU6a-p53, and pAdHM4-hU6b-p53, respectively) into 293 cells as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the original intact hU6 promoter sequence (without a target sequence; Ad-hU6) were similarly constructed with pHM5-hU6 and pAdHM4. The virus was purified by CsCl₂ gradient centrifugation; dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM MgCl₂, and 10% glycerol; and stored in aliquots at –70°C. Determination of virus particle (VP) titers and infectious titers was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and with an Adeno-X rapid titer kit (Clontech), respectively. The infectious titer-to-particle ratio was 1:36 for Ad-hU6, 1:31 for Ad-hU6-Lu, 1:28 for Ad-hU6a-Lu, 1:24 for Ad-hU6b-Lu, 1:22 for Ad-hU6-p53, 1:12 for Ad-hU6a-p53, and 1:15 for Ad-hU6b-p53.

Adenovirus-mediated gene transduction and luciferase assay

A549 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. Determination of luciferase production in the cells and extraction of cellular protein for Western blotting were performed after a 72-hr culture period. Luciferase production in the cells was measured with a luciferase assay system (PicaGene LT 2.0; produced by Toyo Ink [Tokyo, Japan] for Wako [Kyoto, Japan])

Western blotting for p53

Cell extracts were prepared in lysis buffer (25 mM Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured

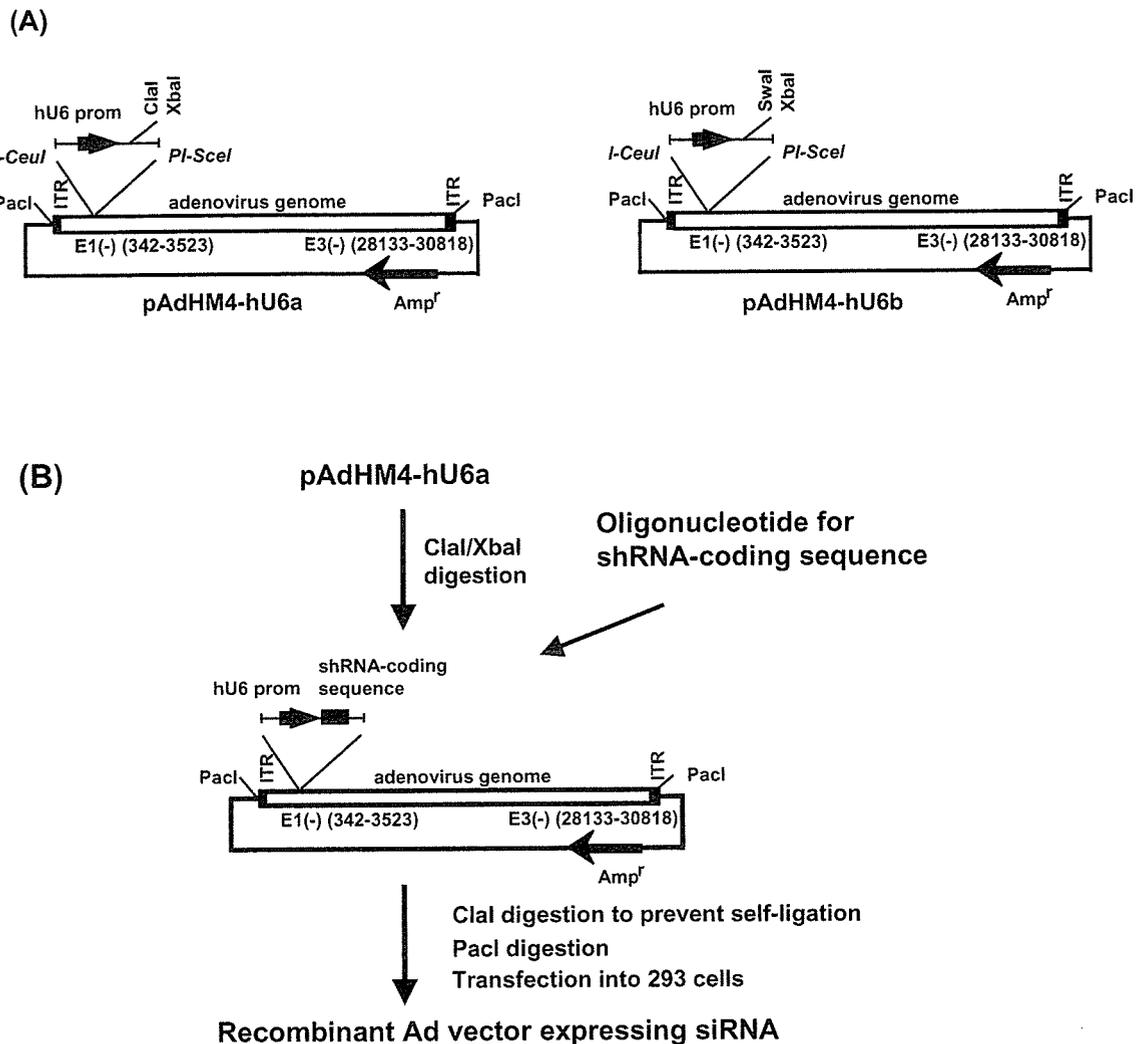


FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. (A) Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *ClaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. pAdHM4-hU6b contains a unique *SwaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. (B) Construction strategy for the Ad vector expressing siRNA. pAdHM4-hU6a was digested with *ClaI/XbaI* and ligated with oligonucleotides for the shRNA-coding sequence. Ligation products were then digested with *ClaI* to prevent the generation of nonrecombinant parental plasmid. The resulting plasmid was linearized by digestion with *PaclI* and transfected into 293 cells, generating recombinant Ad vectors expressing siRNA. pAdHM4-hU6b was similarly used.

with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μ g) were electrophoresed on sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in nonfat dry milk, the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Oncogene Research Products/EMD Biosciences, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products/EMD Biosciences). The filters were developed by chemiluminescence (ECL Western blotting detection sys-

tem; GE Healthcare, Piscataway, NJ). The signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified with Image Gauge software (Fujifilm).

RESULTS AND DISCUSSION

Rapid and efficient construction of Ad vectors expressing siRNA offers the promise of using RNAi in the context of both gene function analysis and therapeutic applications. In the present study, we developed a simple method for constructing Ad vectors expressing siRNA, based on only one-step *in vitro* ligation. To do this, we first constructed an Ad vector plasmid containing the E1- and E3-deleted Ad genome and the hU6 pro-

(A) Intact hU6 promoter

```

      ┌───┐ transcription
GAA ACA CCG
CTT TGT GGC

```

(B) The hU6a promoter (in this study)

```

      ┌───┐ transcription
GAA AAT |cgx
CTT TTA |Gcx
      └───┘
      ClaI

GAA AAT cgx xxx ... .. ttcaagaga xxx ... .. ttttt ggaaa t
CTT TTA GCx xxx ... .. aagttctct xxx ... .. aaaaa ccttt agatc
      target sequence      loop      target sequence      transcription      XbaI site
      (sense)

```

(C) The hU6b promoter (in this study)

```

      ┌───┐ transcription
GAA TTT |xxx
CTT AAA |xxx
      └───┘
      SwaI

GAA TTT ccg xxx ... .. ttcaagaga xxx ... .. ttttt ggaaa t
CTT AAA ggc xxx ... .. aagttctct xxx ... .. aaaaa ccttt agatc
      target sequence      loop      target sequence      transcription      XbaI site
      (sense)

```

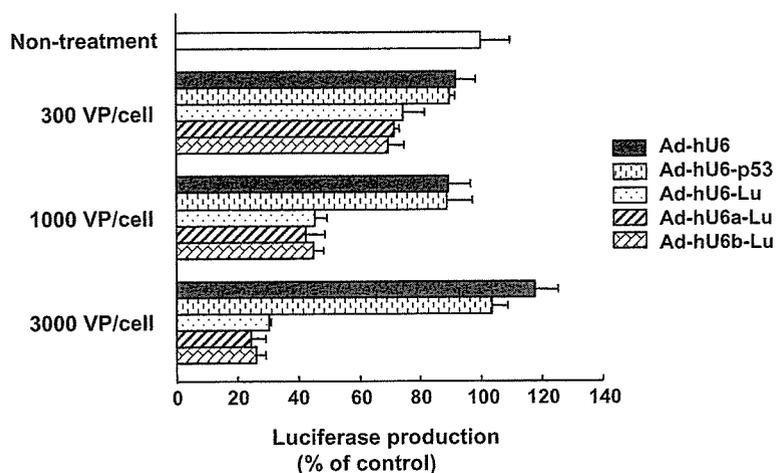
FIG. 2. Sequences at the transcription start site of the new hU6 promoter. (A) Intact hU6 promoter sequence. (B) hU6a promoter sequence. In this promoter, the *ClaI* site is placed at the transcription start site. (C) hU6b promoter sequence. In this promoter, a *SwaI* site is placed at the transcription start site. shRNA-coding oligonucleotides to be synthesized for each promoter are shown as lower-case letters on the right-hand side.

moter sequence in the E1 deletion region, pAdHM4-hU6a and pAdHM4-hU6b (Fig. 1A). By introducing the hU6 promoter sequence into the vector plasmid in advance, the cloning step of the gene of interest from the shuttle plasmid to the vector plasmid, which is an essential step in the conventional method for constructing Ad vectors (namely, the improved *in vitro* ligation method [Mizuguchi and Kay, 1998, 1999] and homologous recombination method in *E. coli* [He *et al.*, 1998]), can be skipped. To make it possible to directly clone the shRNA-coding oligonucleotides downstream of the hU6 promoter sequence, hU6 promoters containing unique restriction enzyme sites at the transcription start site have been developed. The new hU6 promoter sequences contain a *ClaI* or *SwaI* site around the transcription start site and an *XbaI* site downstream from the promoter (Figs. 1 and 2). These enzyme sites were selected because they do not cut the E1- and E3-deleted Ad genome. Because the transcription of shRNA might be influenced by the mutated sequences around the transcription start site, two types of hU6 promoters, differing by only a few nucleotides, were constructed. The hU6a promoter sequence contains a *ClaI* site, whereas the hU6b promoter sequence contains a *SwaI* site. *ClaI*, *SwaI*, and *XbaI* sites are unique in the vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. To generate a recombinant vector plasmid for Ad vectors expressing siRNA, oligonucleotides for shRNA against the target gene were synthesized, annealed, and ligated with *ClaI/XbaI*-digested pAdHM4-hU6a or *SwaI/XbaI*-digested pAdHM4-hU6b. Oligonucleotides were designed so that recombinant vector plasmid containing the shRNA-coding sequence is redigested with *XbaI*, but not with *ClaI* or *SwaI*. By designing oligonucleotides like the one described above, the generation of self-ligated plasmid can be avoided by digestion of the ligation products with *ClaI* or *SwaI*. On the right side of Fig. 2, DNA sequences, including the shRNA-coding sequence around the transcription start site of the hU6 promoter, are shown. Oligonucleotides that must be synthesized for the shRNA-coding sequence are shown as

lower-case letters. By using the method developed in the present study, we could easily generate Ad vectors expressing siRNAs against luciferase and human p53. More than 90% of the recombinant Ad vector plasmids contained the correct insert. Because the *ClaI*- (or *SwaI*-) and *XbaI*-digested pAdHM4-hU6a and pAdHM4-hU6b can be stored at -20°C , only the ligation-based introduction of oligonucleotides into these sites of the vector plasmid would be required for the construction of an appropriate vector.

To examine the function of Ad vectors expressing siRNA against luciferase (Ad-hU6a-Lu and Ad-hU6b-Lu), the efficiency of knockdown of luciferase expression in A549-Luc cells, which stably express luciferase, was examined by treatment with Ad-hU6a-Lu or Ad-hU6b-Lu (Fig. 3). Ad-hU6-Lu, in which the hU6 promoter contains the original intact sequence even after introduction of an shRNA-coding sequence, was used as a positive control. To generate Ad-hU6-Lu, the shRNA-coding sequence was first introduced downstream from the hU6 promoter sequence cloned into the shuttle plasmid, according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); the shRNA expression cassette was then introduced into the E1 deletion region of the Ad vector plasmid pAdHM4 (Mizuguchi and Kay, 1998). Transfection of a *PacI*-digested vector plasmid into 293 cells generated Ad-hU6-Lu. Ad-hU6, which contains the intact hU6 promoter without the shRNA-coding sequence, and Ad-hU6-p53, which contains the intact hU6 promoter with the shRNA-coding sequence against human p53, were similarly constructed and used as negative controls. Data showed that Ad-hU6a-Lu and Ad-hU6b-Lu suppressed luciferase expression in A549-Luc cells as efficiently as Ad-hU6-Lu, in a dose-dependent manner (Fig. 3). Ad-hU6 and Ad-hU6-p53 showed no effects on luciferase expression. Ad-hU6a-p53 and Ad-hU6b-p53 (these Ad vectors are used in Fig. 4) also had no influence on luciferase expression (data not shown). The RNAi effect of luciferase expression was relatively weak compared with that of p53 (de-

FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means and SD ($n = 4$).



scribed below). This difference probably occurred because the A549-Luc cells were expressing luciferase from a strong viral promoter (SV40 promoter and enhancer) and because the levels of luciferase expression were higher than those of endogenous p53 expression.

We next examined the RNAi effect of the siRNA-expressing Ad vector generated in the present study on the endogenous gene. As a model, we silenced p53 expression in A549 cells (Fig. 4). Ad-hU6a-p53 and Ad-hU6b-p53 were generated, and Ad-hU6, Ad-hU6-Lu, and Ad-hU6-p53 were also used. Ad-hU6-p53 contains the intact hU6 promoter sequence, including the transcription start site, even after introduction of the shRNA-coding sequence. A549 cells were transduced with a 300- or 1000-VP/cell of each Ad vector, and cultured for 3 days. Levels of p53 expression were examined by Western blotting. Expression of actin was also measured as an internal control. Expression of p53 in A549 cells was efficiently decreased by treatment with Ad-hU6a-p53 and Ad-hU6b-p53 as well as with Ad-hU6-p53. Levels of p53 expression in cells treated with Ad-hU6-p53, Ad-hU6a-p53, or Ad-hU6b-p53 at 1000 VP/cell were decreased to 7, 2, and 5%, respectively, relative to cells treated with Ad-hU6, according to Image Gauge software (Fujifilm) (in the case of 300 VP/cell, they were decreased to 53, 24, and 30%, respectively). The efficiency of p53 silencing by treatment with Ad-hU6-p53 was slightly lower than that with Ad-hU6a-p53 or Ad-hU6b-p53. This reduced efficiency is likely due to the approximately 1.5 to 2 times lower infectious titer-to-particle ratio of Ad-hU6-p53 in comparison with those of Ad-hU6a-p53 and Ad-hU6b-p53. Ad-hU6 and Ad-hU6-Lu did not decrease the level of p53 expression (Fig. 4). These results indicate that new hU6 promoters containing *ClaI* or *SwaI* sites at the transcription start site should transcribe as efficiently as the original hU6 promoter, and that Ad vectors containing the new hU6 promoters efficiently silence target gene expression. Different vector systems (pAdHM4-hU6a and pAdHM4-hU6b) should be used according to the specific purpose.

To facilitate the construction of an siRNA expression plasmid, the U6 and H1 promoters, which contain *ApaI*, *BbsI*, *BglIII*, *EcoRV*, *SallI*, and *XbaI* sites, etc., at the transcription start site, have been developed (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Boden *et al.*, 2003). All types of promoters

worked efficiently, and could be widely used for efficient RNAi, although the efficiency (activity) of the mutated promoters described above has not been compared with that of the intact promoter. The present study clearly showed that the mutated hU6 promoter, at least one having a *ClaI* or *SwaI* site at the transcription start site and an *XbaI* site downstream of the promoter sequence, is similar in activity to the intact hU6 promoter and would not influence the function of the promoter.

The method using polymerase chain reaction (PCR)-based amplification of shRNA together with the U6 promoter followed by subsequent cloning of the complete expression cassette directly into the Ad vector genome is another strategy for one-step construction of recombinant Ad plasmids containing an siRNA expression cassette. In this method, however, the procedures described below are required for preparation of insert DNA: (1) ordering of the PCR primer, (2) PCR, (3) purification of the PCR product, (4) restriction enzyme digestion and purification of the PCR product, and (5) ligation. In our present system, only the following procedures are required: (1) ordering of the oligonucleotides, (2) hybridization of the oligonucleotides, and (3) ligation. Thus, the present method would be much easier and would allow any laboratory to easily construct

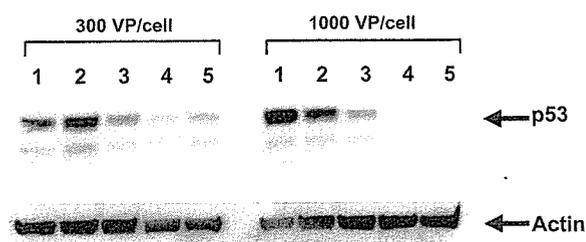


FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted from the cells, and the levels of p53 expression were examined by Western blotting. The actin bands served as an internal control for equal total protein loading. The extra (lower) bands of p53 are nonspecific.

Ad vectors expressing siRNA for gene transfer studies and therapeutic applications.

Various types of promoters that are based on polymerase II as well as polymerase III have been developed to transcribe shRNA (siRNA) (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). Although the present study applied the most commonly used U6 promoter for simple and efficient construction of siRNA-expressing Ad vectors, this method could easily be applied to vectors using other promoters including polymerase II-based promoters. This method can also easily be combined with various types of improved Ad vectors, such as Ad vectors containing capsid modification (Koizumi *et al.*, 2003, 2006; Mizuguchi and Hayakawa, 2004; Kurachi *et al.*, 2006) or Ad vectors belonging to different subgroups to modify tropism (Sakurai *et al.*, 2003), and Ad vectors containing a tetracycline-inducible RNAi system (Hosono *et al.*, 2004). The method developed in the present study should be a powerful tool for the application of RNAi, and might facilitate the development of an siRNA-expressing Ad vector library for functional screening.

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Fiber-Modified Adenovirus Vectors Decrease Liver Toxicity through Reduced IL-6 Production¹

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Adenovirus (Ad) vectors are one of the most commonly used viral vectors in gene therapy clinical trials. However, they elicit a robust innate immune response and inflammatory responses. Improvement of the therapeutic index of Ad vector gene therapy requires elucidation of the mechanism of Ad vector-induced inflammation and cytokine/chemokine production as well as development of the safer vector. In the present study, we found that the fiber-modified Ad vector containing poly-lysine peptides in the fiber knob showed much lower serum IL-6 and aspartate aminotransferase levels (as a maker of liver toxicity) than the conventional Ad vector after i.v. administration, although the modified Ad vector showed higher transgene production in the liver than the conventional Ad vector. RT-PCR analysis showed that spleen, not liver, is the major site of cytokine, chemokine, and IFN expression. Splenic CD11c⁺ cells were found to secrete cytokines. The tissue distribution of Ad vector DNA showed that spleen distribution was much reduced in this modified Ad vector, reflecting reduced IL-6 levels in serum. Liver toxicity by the conventional Ad vector was reduced by anti-IL-6R Ab, suggesting that IL-6 signaling is involved in liver toxicity and that decreased liver toxicity of the modified Ad vector was due in part to the reduced IL-6 production. This study contributes to an understanding of the biological mechanism in innate immune host responses and liver toxicity toward systemically administered Ad vectors and will help in designing safer gene therapy methods that can reduce robust innate immunity and inflammatory responses. *The Journal of Immunology*, 2007, 178: 1767–1773.

Recombinant adenovirus (Ad)³ vectors are widely used for gene therapy experiments and clinical gene therapy trials. One of the limitations of Ad vector-mediated gene transfer is the immune response after systemic administration of the Ad vector (1, 2). The immune response to the Ad vector and Ad vector-transduced cells dramatically affects the kinetics of the 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. The immunogenic toxicities associated with the use of Ad vectors involve both innate and adaptive immune responses.

In the first generation Ad vector lacking the *E1* gene, leaky expression of viral genes from the vector stimulates an immune response against the Ad vector-transduced cells (3–5). The CTL response can be elicited against viral gene products and/or transgene products expressed by transduced cells. The molecular mechanism of this toxicity

has been studied extensively, and the helper-dependent (guttled) Ad vector, which deletes all of the viral protein-coding sequences, has been developed to overcome this limitation (6–8). The humoral virus-neutralizing Ab responses against the Ad capsid itself are another limitation, preventing transgene expression upon the subsequent administration of vectors of the same serotype. Because hexons are mainly targeted by neutralizing Abs, hexon modification has been reported to allow for escape from neutralizing Abs (9). The Ad vectors belonging to types of the subgroup other than Ad type 5, including an Ad type 11- or 35-based vector, or to species other than human have also been developed (10–13).

Regarding the innate immune response, shortly after systemic injection of the Ad vector cytokines/chemokines are produced and an inflammatory response occurs in response to the Ad vector and Ad vector-transduced cells. It has been reported that activated Kupffer cells (and monocytes and resident macrophages) and dendritic cells (DC) release proinflammatory cytokines/chemokines such as IL-6, TNF- α , IP-10, and RANTES, causing the activation of an innate immune response (14, 15). NF- κ B activation is likely to play a central role in inflammatory cytokine/chemokine production (16, 17). Although many papers regarding the innate immune response to the Ad vector have been published thus far, the biological mechanism has not been clearly elucidated. Even the cell types responsible for the innate immune response have not been identified. Understanding the mechanism of and identifying the cell types responsible for the innate immune response and liver inflammation are crucial to the construction of new vectors that are safer and efficiently transduce target tissue. Modification of the Ad vector with polyethylene glycol (PEG) reduces the innate immune response and also prolongs persistence in the blood and circumvents neutralization of the Ad vectors by Abs (18–21). We have previously reported that the mutant Ad vector ablating coxsackievirus and Ad receptor (CAR) (the first receptor) binding, α , integrin (the secondary receptor) binding, and heparan sulfate glycosaminoglycan (HSG) (the third receptor) binding reduced (or blunted)

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³ Abbreviations used in this paper: Ad, adenovirus; AST, aspartate aminotransferase; CAR, coxsackievirus and Ad receptor; DC, dendritic cell; HSG, heparan sulfate glycosaminoglycan; PEG, polyethylene glycol; VP, virus particle.

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liver toxicity and IL-6 production (22). However, these two Ad vectors mediate significantly lower tissue transduction due to steric hindrance by PEG chains and a loss of binding activity to the receptor, respectively (20–22). An Ad vector showing efficient transduction and reduced innate immune response has not yet been developed.

In the present study, we elucidate the molecular mechanism of the innate immune response by the Ad vector and characterize the safer Ad vector, which reduces the innate immune response and liver toxicity. We found that the fiber-modified Ad vector containing a stretch of lysine residues (K7 (KKKKKKK) peptide) (23–25) that target heparan sulfates on the cellular surface greatly reduced IL-6 and liver toxicity after i.v. injection into mice compared with the conventional Ad vector. IL-6 and the other immune cytokines, chemokines, and IFNs were mainly produced from the spleen and especially from conventional DC (CD11c⁺B220⁻ cells), not the liver. The spleen distribution of the K7-modified Ad vector was reduced compared with the conventional Ad vector. The K7-modified Ad vector decreased the liver toxicity (aspartate aminotransferase (AST) levels), at least in part due to the reduced serum IL-6 levels. Importantly, this K7-modified Ad vector maintained high transduction efficiency *in vivo* and showed somewhat higher transgene production in the liver than a conventional Ad vector.

Materials and Methods

Ad vector

Two luciferase-expressing Ad vectors, Ad-L2 and AdK7-L2, have been constructed previously (25, 26). The CMV promoter-driven luciferase gene derived from the pGL3-Control was inserted into the E1 deletion region of the Ad genome. Ad-L2 contains wild-type fiber, whereas AdK7-L2 contains the polylysine peptide KKKKKKK in the C-terminal of the fiber knob (25). Viruses (Ad-L2 and AdK7-L2) were prepared as described previously (25) and purified by CsCl₂ step gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically by the method of Maizel et al. (27).

Ad-mediated transduction *in vivo*

Ad-L2 or AdK7-L2 were i.v. administered to C57BL6 mice (1.0×10^{10} virus particles (VP)) (6-wk-old males obtained from Nippon SLC). Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated and homogenized as previously described (28). Luciferase production was determined using a luciferase assay system (PicaGene 5500; Toyo Inki). Protein content was measured with a Bio-Rad assay kit using BSA as a standard.

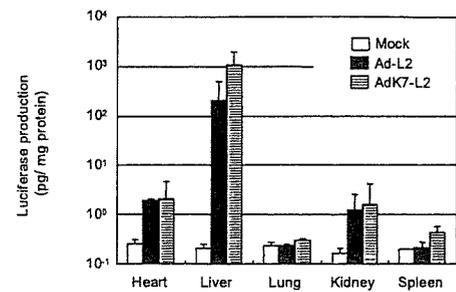
The amounts of Ad genomic DNA in the each organ were quantified with the TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; PerkinElmer Applied Biosystems). Samples were prepared with DNA templates isolated from each organ (25 ng) by an automatic nucleic acid isolation system (NA-2000; Kurabo Industries). The amounts of Ad DNA were quantified with the TaqMan fluorogenic detection system (PerkinElmer Applied Biosystems) as described in our previous report (22).

To analyze the involvement of IL-6 signaling in liver toxicity in response to Ad vector administration, 100 μ g per mouse of an anti-IL-6R Ab (clone D7715A7; BioLegend) that specifically blocks IL-6 signaling was i.p. administered to C57BL6 mice 1.5 h before Ad-L2 administration (3.0×10^{10} VP). Rabbit IgG (clone R3-34; BD Biosciences) was administered as a control. Serum samples and liver tissue were collected 48 h later, and AST levels in the serum and luciferase production in the liver were determined.

Liver serum enzymes and cytokine levels after systemic administration

Blood samples were collected by the inferior vena cava at the indicated times (3 or 48 h) after i.v. administration of Ad-L2 or AdK7-L2 (3.0×10^{10} and 1.0×10^{11} VP, respectively). IL-6 and IL-12 levels in serum samples collected at 3 h after Ad injection were measured by an ELISA kit (BioSource International). The levels of AST in serum samples collected at 24 and 48 h were measured with the Transaminase-CII kit (Wako Pure Chemical). Forty-eight hours after the Ad vector injection, the mice were killed and their livers were collected. The liver was washed, fixed in 10% formalin, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with H&E. This process was commissioned to the Applied Medical Research Laboratory (Osaka, Japan).

A



B

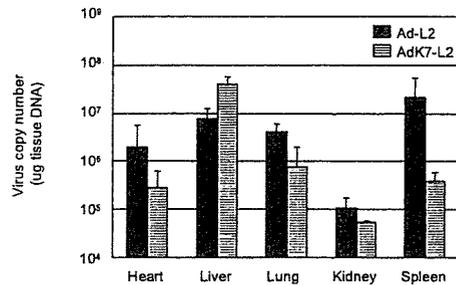


FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production (A) and Ad vector DNA (B) in each organ were measured by a luciferase assay system or the quantitative TaqMan PCR assay, respectively. All data represent the means \pm SD of 4–6 mice.

Cytokines and chemokines mRNA levels in tissue after systemic administration

Total tissue RNA samples were isolated by the reagent ISOGEN (Wako Pure Chemical) 3 h after the i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). Reverse transcription was performed using the SuperScript first-strand synthesis system for first-strand cDNA synthesis (Invitrogen Life Technologies) according to the instructions of the manufacturer. IL-6 and IL-12 mRNA in the liver and spleen were quantified with the TaqMan fluorogenic detection system (PerkinElmer Applied Biosystems). Semiquantified RT-PCR analysis was also performed to determine mRNA levels of the cytokines, chemokines, and IFNs (total eight mRNA). The primer sequences and probes were as follows: IL-6 forward, 5'-GAG GAT ACC ACT CCC AAC AGA CC-3'; IL-6 reverse, 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3' (reverse); IL-6 probe, 5'-CAG AAT TGC CAT TGC ACA ACT CTT TTC TCA-3'; IL-12p40 forward, 5'-GGA AGC ACG GCA GCA GAA TA-3'; IL-12p40 reverse, 5'-AAC TTG AGG GAG AAG TAG GAA TGG-3'; IL-12p40 probe, 5'-CAT CAT CAA ACC AGA CCC GCC CAA-3'; TNF- α forward, 5'-CCT GTA GCC CAC GTC GTA GC-3'; TNF- α reverse, 5'-TTG ACC TCA GCG CTG AGT TG-3'; RANTES forward, 5'-ATG AAG ATC TCT GCA GCT GCC CTC ACC-3'; RANTES reverse, 5'-CTA GCT CAT CTC CAA ATA GTT GAT G-3'; MIP-2 forward, 5'-ACC TGC CGG CTC CTC AGT GCT GC-3'; MIP-2 reverse, 5'-GGC TTC AGG GTC AAG GCA AAC-3'; IFN- α forward, 5'-AGG CTC AAG CCA TCC CTG T-3'; IFN- α reverse, 5'-AGG CAC AGG GGC TGT CTT TCT TCT-3'; IFN- β forward, 5'-TTC CTG CTG TGC TTC TCC AC-3'; IFN- β reverse, 5'-GAT TCA CTA CCA GTC CCA GAG TC-3'; IFN- γ forward, 5'-GAG GAT ACC ACT CCC AAC AGA CC-3'; IFN- γ reverse, 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'; GAPDH forward, 5'-TTC ACC ACC ATG GAG AAG GC-3'; and GAPDH reverse, 5'-GGC ATG GAC TGT GGT CAT GA-3'. The expected sizes of the PCR products are as follows: IL-6, 193 bp; IL-12p40, 155 bp; TNF- α , 374 bp; RANTES, 252 bp; MIP-2, 221 bp; IFN α , 272 bp; IFN β , 607 bp; IFN- γ , 306 bp; and GAPDH, 237 bp.