peripheral T cells when challenged through the T-cell receptor. However, it has been reported that PKCλ-deficient mice have a lethal phenotype at the early embryonic stage (Soloff et al., 2004). Based on the present results and those of previous reports (Kanayasu-Toyoda et al., 1999, 2002), we postulate that PKCι plays an important role in regulating G-CSF-induced proliferation in neutrophilic lineage cells.

Acknowledgments

We thank the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) for their kind cooperation. This work was supported in part by a grand-in-aid for health and labor science research (H17-SAISEI-021) from the Japanese Ministry of Health, Labor and Welfare, and in part by a grand-in-aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

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HUMAN GENE THERAPY 18:74–80 (January 2007)
© Mary Ann Liebert, Inc.
DOI: 10.1089/hum.2006.129

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 homologies 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 promoter 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 stain 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/*Sal*I fragment of piGene hU6 (iGENE Therapeutics, Tsukuba, Japan), was also introduced into the *Sph*I and *Sal*I sites of pHM5 (Mizuguchi and Kay, 1999), resulting in pHM5-ihU6. pHM5-ihU6 was then digested with *Sal*I 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	aaggtcgggcaggaagagggccta
hU6-AS1 primer	ggtctagaagtatcgatttcgtctttccacaagatatat (*Xba*I and *Cla*I recognition sequences are underlined and italicized, respectively)
hU6-AS2 primer	ggtctagaagtatttaaattcgtcctttccacaagatatataa (*Xba*I and *Swa*I recognition sequences are underlined and italicized, respectively)
Oligonucleotide 1	cgacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgtttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 2	ctagatttccaaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 3	ccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgtttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 4	ctagatttccaaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgtgg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 5	cggactccagtggtaatctacttcaagagagtagattaccactggagtctttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 6	ctagatttccaaaaagactccagtggtaatctactctcttgaagtagattaccactggagtc (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 7	ccgactccagtggtaatctacttcaagagagtagattaccactggagtctttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 8	ctagatttccaaaaagactccagtggtaatctactctcttgaagtagattaccactggagtcgg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 9	tcgacctgcaggcatgcaagcttc (BspMI recognition sequences are underlined)
Oligonucleotide 10	ctaggaagcttgcatgcctgcagg (BspMI recognition sequences are underlined)
Oligonucleotide 11	caccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgtttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 12	gcataaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 13	caccgactccagtggtaatctacttcaagagagtagattaccactggagtcttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 14	gcataaaaagactccagtggtaatctactctcttgaagtagattaccactggagtc (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 $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, 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 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured

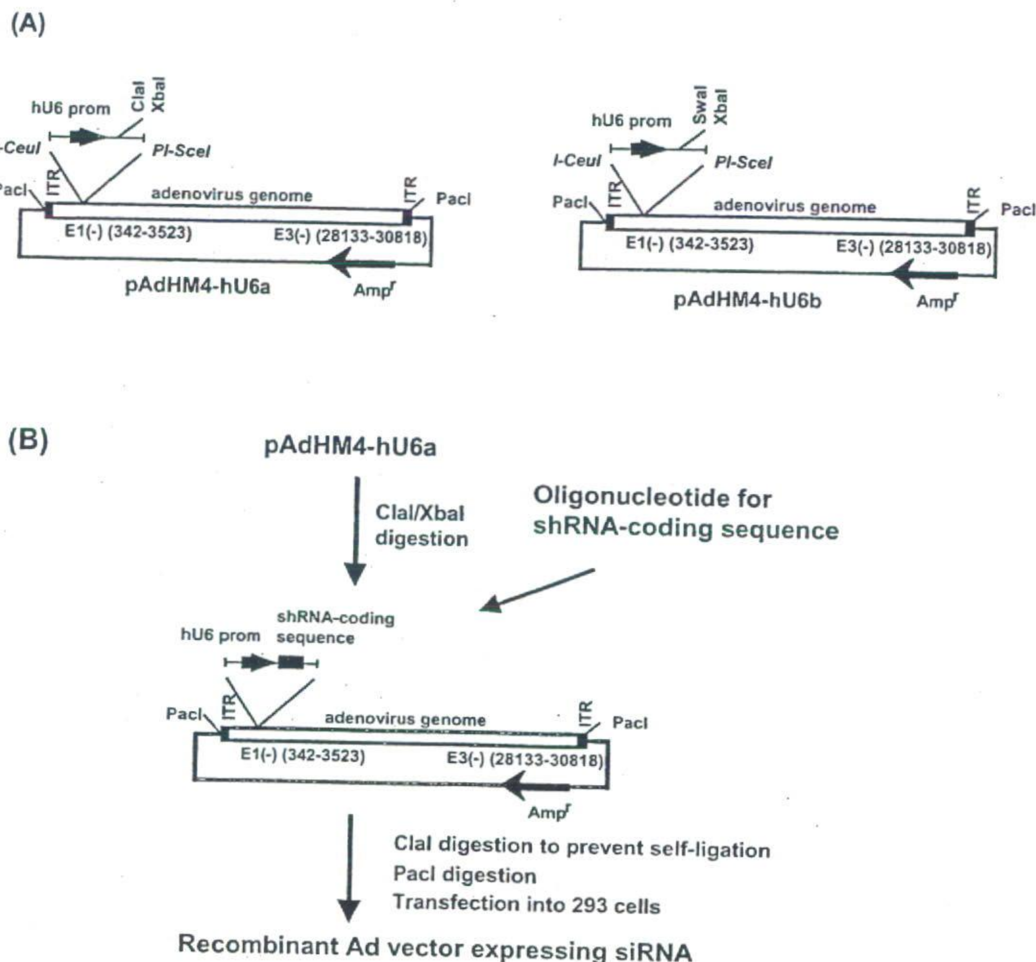

FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. **(A)** Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *Cla*I site at the transcription start site of the hU6 promoter sequence and an *Xba*I site downstream from the promoter sequence. pAdHM4-hU6b contains a unique *Swa*I site at the transcription start site of the hU6 promoter sequence and an *Xba*I site downstream from the promoter sequence. **(B)** Construction strategy for the Ad vector expressing siRNA. pAdHM4-hU6a was digested with *Cla*I/*Xba*I and ligated with oligonucleotides for the shRNA-coding sequence. Ligation products were then digested with *Cla*I to prevent the generation of nonrecombinant parental plasmid. The resulting plasmid was linearized by digestion with *Pac*I 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 μg) 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 system; 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)

```
GAA AAT|cgx        GAA AAT cgx xxx ··· ··· ttcaagaga xxx ··· ··· ttttt ggaaa t
CTT TTA GC|x       CTT TTA GCx xxx ··· ··· aagttctct xxx ··· ··· aaaaa ccttt agatc
   ClaI
```

transcription; target sequence (sense); loop; target sequence (anti-sense); transcription stop; XbaI site

(C) The hU6b promoter (in this study)

```
GAA TTT|xxx        GAA TTT ccg xxx ··· ··· ttcaagaga xxx ··· ··· ttttt ggaaa t
CTT AAA|xxx        CTT AAA ggc xxx ··· ··· aagttctct xxx ··· ··· aaaaa ccttt agatc
   SwaI
```

transcription; target sequence (sense); loop; target sequence (anti-sense); transcription stop; XbaI site

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 *Cla*I site is placed at the transcription start site. **(C)** hU6b promoter sequence. In this promoter, a *Swa*I 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 *Cla*I or *Swa*I site around the transcription start site and an *Xba*I 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 *Cla*I site, whereas the hU6b promoter sequence contains a *Swa*I site. *Cla*I, *Swa*I, and *Xba*I 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 *Cla*I/*Xba*I-digested pAdHM4-hU6a or *Swa*I/*Xba*I-digested pAdHM4-hU6b. Oligonucleotides were designed so that recombinant vector plasmid containing the shRNA-coding sequence is redigested with *Xba*I, but not with *Cla*I or *Swa*I. By designing oligonucleotides like the one described above, the generation of self-ligated plasmid can be avoided by digestion of the ligation products with *Cla*I or *Swa*I. 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 *Cla*I- (or *Swa*I-) and *Xba*I-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 *Pac*I-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-

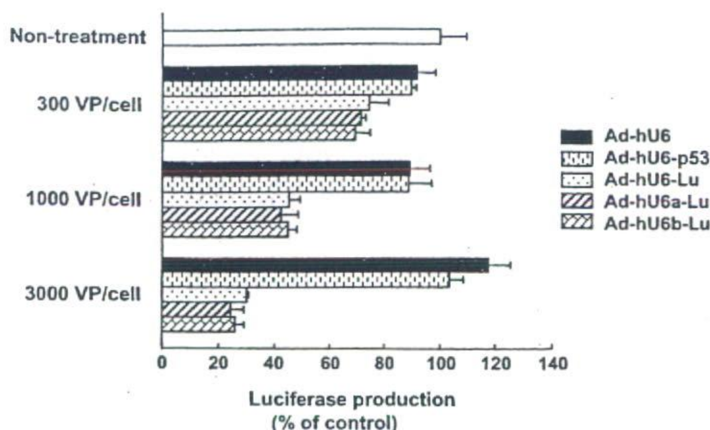

FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the 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 titer 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 *Cla*I or *Swa*I 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 *Apa*I, *Bbs*I, *Bgl*II, *Eco*RV, *Sal*I, and *Xba*I 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 *Cla*I or *Swa*I site at the transcription start site and an *Xba*I 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

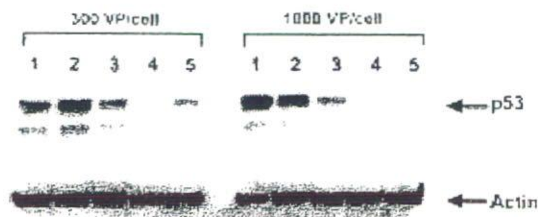

FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted 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.

ACKNOWLEDGMENTS

The authors thank Risako Nagahashi-Nakano and Tomomi Sasaki for technical assistance. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

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Received for publication September 3, 2006; accepted after revision November 12, 2006.

Published online: December 22, 2006.

Fiber-Modified Adenovirus Vectors Decrease Liver Toxicity through Reduced IL-6 Production[1]

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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 secret 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 (gutted) 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-κB 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_v integrin (the secondary receptor) binding, and heparan sulfate glycosaminoglycan (HSG) (the third receptor) binding reduced (or blunted)

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Received for publication August 29, 2006. Accepted for publication November 10, 2006.

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[1] This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

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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 $CsCl_2$ 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 homogenated 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 μg 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 cave 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).

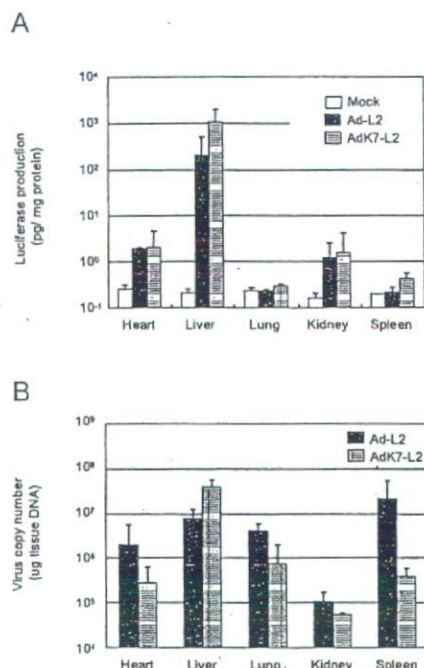

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production (*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 ± 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; IFNα, 272 bp; IFNβ, 607 bp; IFN-γ, 306 bp; and GAPDH, 237 bp.

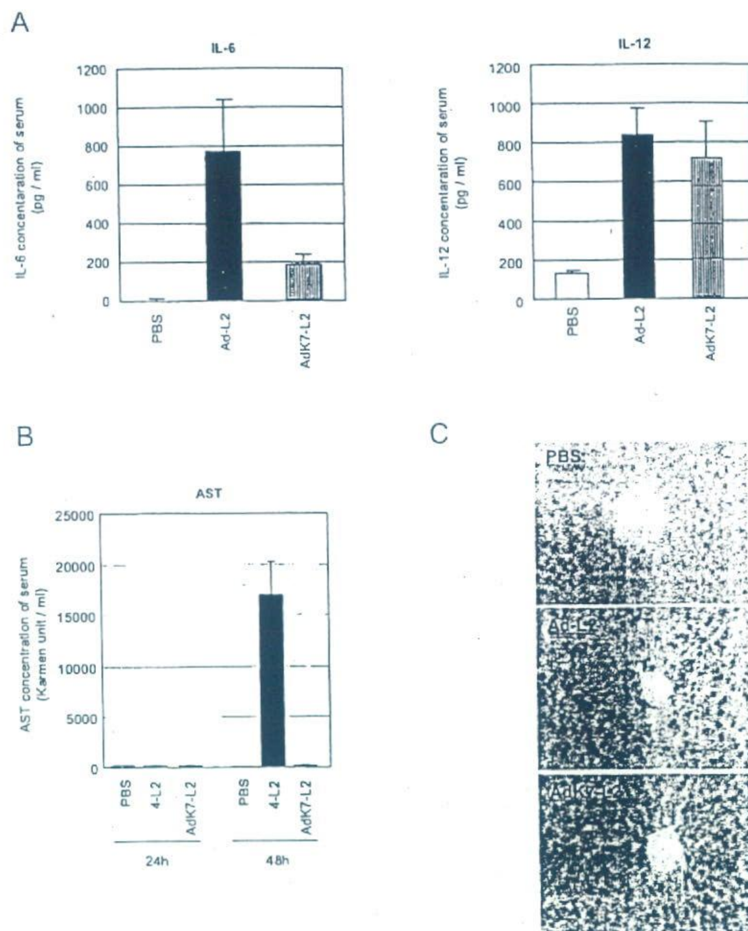

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*). The livers were collected after 48 h following the injection (3.0×10^{10} VP) (*C*). *A*, IL-6 and IL-12 levels in the serum were measured by ELISA. *B*, AST levels in the serum were measured using a Transaminase-CII kit. *C*, Paraffin sections of the livers were prepared. Each section was stained with H&E. Data represent the means ± SD of four mice.

Cell sorting of splenic cells

Splenic conventional DC, plasmacytoid DC, and B cells, which were $CD11c^{+}B220^{-}$, $CD11c^{+}B220^{+}$, and $CD11c^{-}B220^{+}$ cells, respectively, were sorted by FACS Aria (BD Biosciences). Total RNA samples were isolated from each cell by the reagent ISOGEN, and RT-PCR analysis was then performed as described above.

Results

This study was undertaken to elucidate the biological mechanism in the innate immune host responses toward i.v. administered Ad vector. The relationship between the innate immune response and liver toxicity by systemic administration of the Ad vectors was also examined.

Gene transduction and Ad vector accumulation in vivo

In this study we used the conventional Ad vector (Ad-L2) and a fiber-modified Ad vector containing a polylysine (K7) peptide (AdK7-L2), both of which express luciferase under the control of the CMV promoter. First, we examined luciferase production in the organ and the biodistribution of viral DNA after i.v. administration of AdK7-L2 (1.0×10^{10} VP) into mice compared with Ad-L2 (see Fig. 3). The vector dose of 1.0×10^{10} VP was selected because this dose did not induce any apparent toxicity (IL-6 and AST production) with either Ad-L2 or AdK7-L2. When a higher dose (3.0×10^{10} or 1.0×10^{11} VP) was used, only Ad-L2 and not AdK7-L2 showed toxicity (described later), which does not reflect an exact comparison of the transduction efficiency. The Ad type 5-based vector delivers the foreign gene predominantly in the liver after i.v. injection into mice (29, 30). Interestingly, AdK7-L2 mediated ~6-fold higher liver transduction than Ad-L2 (Fig. 1*A*). In contrast, the luciferase production in the heart, lung, kidney, and spleen in response to AdK7-L2 was similar to that in response to Ad-L2. To examine the biodistribution of Ad-L2 and AdK7-L2 in mice, the amounts of Ad DNA in each organ 48 h after the injection of Ad vectors were measured with the TaqMan fluorogenic detection system. More AdK7-L2 DNA accumulated in the liver than Ad-L2 DNA (Fig. 1*B*), although the amounts of AdK7-L2 DNA in the heart, lung, kidney, and spleen were less than those of Ad-L2 DNA. In particular, the amounts of AdK7-L2 DNA in the spleen were ~56-fold less than those of Ad-L2 DNA. The data regarding luciferase production (Fig. 1*A*) and the amounts of Ad DNA in most organs (Fig. 1*B*) showed discrepancies. Luciferase production in the liver was >2 log order higher than that in other organs, while the amounts of Ad DNA in liver were not as striking among the organs compared with luciferase production. This difference is likely due to the difference in the amount of nonspecific viral uptake among the organs. Reduced spleen accumulation of AdK7-L2 DNA, compared with Ad-L2 DNA, was also observed at a dose of 1.0×10^{11} VP (data not shown).

Serum cytokines and AST levels

The systemic administration of Ad vectors results in the initiation of strong innate immune responses and inflammation in animals and humans (1), and this toxicity limits the utility of Ad vectors for gene therapy. To evaluate the innate immune response and liver toxicity of each Ad vector, we measured the levels of IL-6, IL-12, and AST in serum. Because IL-6 in the serum and hepatic toxicity

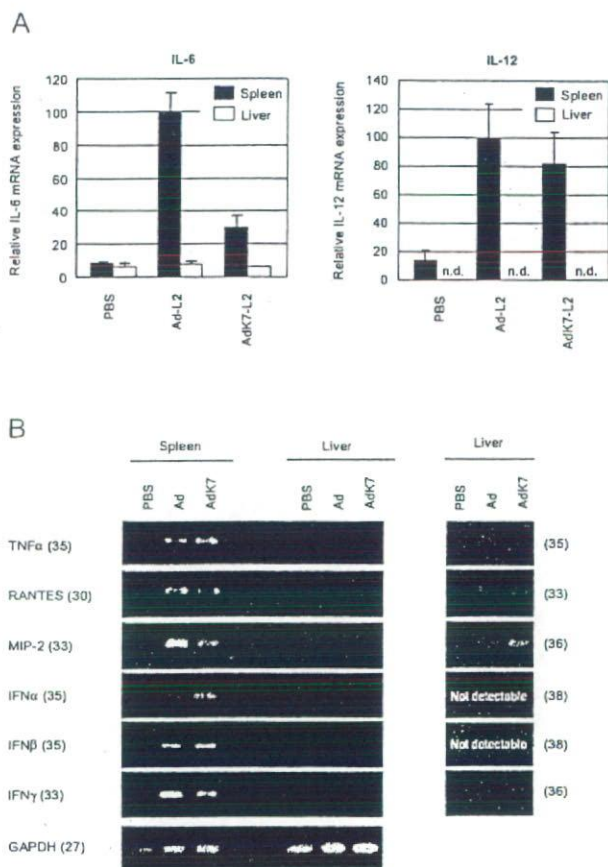

FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, IL-6 and IL-12 cDNA were measured with the quantitative TaqMan PCR assay (*A*). The expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ was measured by semiquantitative RT-PCR assay (*B*). All data represent the means $\pm$ SD of four mice. Cycle number is given in parentheses.

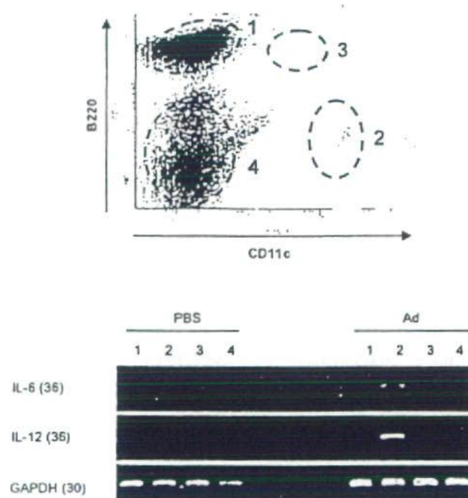

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by RT-PCR assay. *Lane 1*, B cell ($B220^+CD11c^-$); *lane 2*, conventional DC ($B220^-CD11c^+$); *lane 3*, plasmacytoid DC ($B220^+ CD11c^+$); *lane 4*, other cells ($B220^-CD11c^-$). Cycle number is given in parentheses.

analysis was detected at a dose of $>1.0 \times 10^{11}$ or 3.0×10^{10} VP, respectively, these doses were used.

IL-6 levels in response to AdK7-L2 were one-fourth of those with Ad-L2 (Fig. 2*A*). In contrast, there was no difference in serum IL-12 levels between Ad-L2 and AdK7-L2. Thus, IL-6 and IL-12 appear to be produced by a different mechanism. TNF-α in the serum after the injection of Ad-L2 or AdK7-L2 could not be detected (data not shown). Ad-L2 led to high levels of serum AST at 48 h after injection, while AdK7-L2 did not induce AST (Fig. 2*B*). At 24 h, neither Ad-L2 nor AdK7-L2 induced AST. In histological analysis, degranulation or denucleation occurred in hepatocytes from Ad-L2, while AdK7-L2 did not induce hepatocyte toxicity (Fig. 2*C*). The results using AdK7-L2 were similar to those in the untreated mice (Fig. 2, *B* and *C*), suggesting that AdK7-L2 does not show any liver toxicity. These results suggest that AdK7-L2 shows less IL-6 production and almost no liver toxicity.

Cytokines mRNA levels in liver and spleen cells

Ad vectors induce the expression of various cytokines and chemokines in the innate immune responses by effector cells such as macrophages and DC (15, 17, 31–33). Liver and spleen are two major organs responsible for the location of immune cells. We attempted to determine which organ (liver or spleen) produces cytokines, chemokines, and IFNs (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFNα, IFNβ, and IFN-γ) by quantitative real-time RT-PCR or semiquantitative RT-PCR analysis. IL-6 and IL-12 mRNA levels were not induced in the liver after i.v. administration of Ad vectors (Fig. 3*A*). This result was also checked by the result that specific IL-6 and IL-12 mRNA bands were not detected in the liver by RT-PCR analysis (data not shown). Expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ mRNA was also detected mainly in the spleen, not the liver (Fig. 3*B*). IL-6, MIP-2, and IFN-γ mRNA levels in the spleen in response to AdK7-L2 were lower than those in response to Ad-L2. In the liver, TNF-α, RANTES, MIP-2, and IFN-γ mRNA were detected by a high cycle number of PCR after Ad (Ad-L2 or AdK7-L2) injection, whereas IFN-α and IFN-β could be not detected (Fig. 3*B*).

We next identified the cell types responsible for the IL-6 and IL-12 expression in the spleen after i.v. administration of the Ad vector (Ad-L2). Spleen cells were sorted by FACS Aria based on the expression of CD11c and B220 in conventional DC ($CD11c^+B220^-$), plasmacytoid DC ($CD11c^+B220^+$), and B cells ($CD11c^-B220^+$ cells). IL-6 and IL-12 mRNA were mainly detected in the splenic conventional DC. Only a faint band of IL-12 mRNA was also detected in the splenic plasmacytoid DC ($CD11c^+B220^+$) (Fig. 4). These results suggest that splenic conventional DC are major effector cells of innate immune response (at least IL-6 and IL-12 production) against systemically administered Ad vectors.

Elimination of IL-6 signaling reduces liver toxicity

It has previously been shown that TNF-α is likely to be involved in host responses to Ad vectors in vitro and in vivo (34). Recently, Shayakhmetov et al. (35) have reported that IL-1 signaling, not TNF-α signaling, is involved in Ad vector-associated liver toxicity after i.v. administration. However, the mechanism of liver toxicity

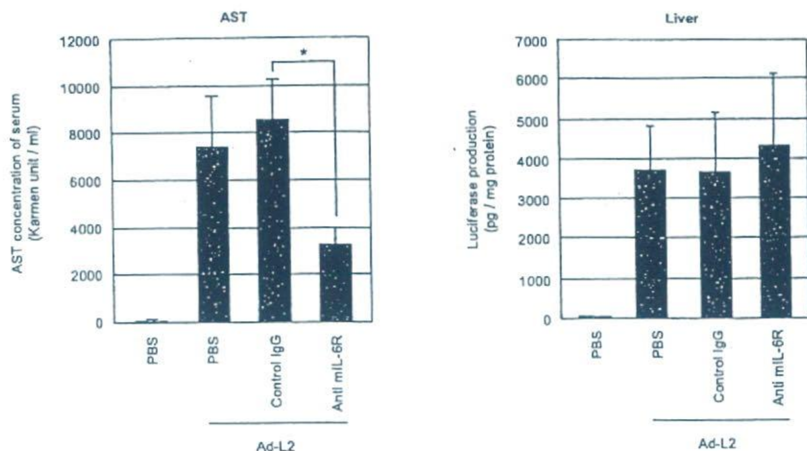

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; R3-34). Ad-L2 or AdK7-L2 (3.0×10^{10} VP) was i.v. injected into the mice 1.5 h later. Blood samples and liver tissue were collected 48 h after the injection of Ad-L2. The AST levels in the serum were measured using a Transaminase-CII kit. Luciferase production in the liver was measured by a luciferase assay system. All data represent the means ± SD of three to four mice. *, $p < 0.01$.

after i.v. Ad administration is poorly understood. In the present study, although AdK7-L2 mediated higher luciferase expression and a higher accumulation of viral DNA in the liver than Ad-L2, it remains unclear why AdK7-L2 showed almost background levels of liver toxicity while Ad-L2 showed high toxicity. As reported previously, inflammatory cytokines, chemokines, and IFNs could be the mediators responsible for liver toxicity (2). IL-6 levels in the serum were the most strikingly different between AdK7-L2 and Ad-L2. Furthermore, IL-6 stimulated acute phase protein (serum amyloid A, fibrinogen, α_1-anti-trypsin, and α_1-acid glycoprotein) in rat and human hepatocytes (36, 37). Therefore, we next examined the effects of serum IL-6 on liver toxicity (Fig. 5). To do this, we used an anti-IL-6R Ab that inhibits the signal through the IL-6 receptor. The IL-6 receptor system consists of two functional molecules, an 80-kDa ligand-binding chain (IL-6R) and a 130-kDa nonligand-binding but signal-transducing chain (gp130). The anti-IL-6R Ab blocks the binding of IL-6 to the IL-6R (38, 39). The anti-IL-6R Ab or the control Ab was i.p. injected 1.5 h before the injection of Ad-L2. The AST levels in the serum and luciferase production in the liver were determined 48 h later. Administration of anti-IL-6R Ab significantly (~2-fold) reduced Ad vector-mediated AST levels in the serum compared with PBS or the control Ab (Fig. 5*A*). Importantly, anti-IL-6R Ab injection did not interfere with luciferase production in the liver (Fig. 5*B*). These results suggest that IL-6 signaling is involved in liver toxicity after i.v. administration of an Ad vector.

Discussion

In this study we found that the fiber-modified Ad vector containing the K7 peptide, which has high affinity with heparin sulfate, shows much lower serum IL-6 and liver toxicity than the conventional Ad vector. This improved characteristic is likely involved with the reduced biodistribution of the vector to the spleen compared with that of the conventional Ad vector. RT-PCR analysis showed that the spleen, not the liver, is the major site of cytokine, chemokine, and IFN (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ) production and that splenic conventional DC are the major effector cells of the innate immune response (at least IL-6 and IL-12 production) after i.v. administration of Ad vectors. We also showed that IL-6 signaling is involved in part with liver toxicity in response to Ad vectors. Importantly, this fiber-modified Ad vector containing the K7 peptide maintained higher transduction efficiency in all the organs examined, and the liver transduction was higher than that of the conventional Ad vector. Although there have been some reports that modified Ad vectors such as the pegylated Ad vector (18–21), the Ad vector containing the Ad type 35 fiber shaft and knob (40), and the triple mutant Ad vector with ablation of CAR, α_v integrin, and HSG binding (22) show decreased innate immune response and liver toxicity, these types of vector lose their transduction activity in vivo. To our knowledge, this is the first report of an Ad vector that maintains high transduction efficiency in vivo with reduced toxicity.

The fiber-modified Ad vector containing the K7 peptide has been developed to overcome the limitations imposed by the CAR dependence of Ad infection. Expanded and efficient gene transfer has been reported based on the use of mutant fiber proteins containing a stretch of lysine residues (23–25). However, there has been no report on the difference in gene transfer activity and toxicity in vivo between the conventional Ad vector and the fiber-modified Ad vector containing the K7 peptide. We have demonstrated that the fiber-modified Ad vector containing the K7 peptide mediates ~6-fold higher mouse liver transduction in response to i.v. administration than the conventional Ad vector (Fig. 1*A*). The amounts of fiber-modified Ad vector DNA in the liver after i.v. administration were also 5-fold higher than those with the conventional Ad vector (Fig. 1*B*). It has been reported that the interaction between the Ad type 5 fiber and the HSG of a hepatocyte is involved in the accumulation in the mouse liver and the cynomolgus monkey liver of systemically administered Ad vectors (41, 42). This fiber-modified Ad vector might mediate more efficient gene transduction through a much higher affinity for HSG. In contrast, the amounts of fiber-modified Ad vector DNA in the spleen after i.v. administration were 56-fold lower than those of the conventional Ad vector (Fig. 1*B*). Biodistribution of viral DNA reflects the total of receptor-mediated uptake and nonspecific uptake. Luciferase production in the cells mainly reflects receptor-mediated uptake. We previously reported that most Ad DNAs are taken up in the liver nonparenchymal cells, not parenchymal cells, after i.v. administration (22). In this study, the conventional Ad vector would also be taken up in the macrophages and DC by nonspecific uptake, resulting in significantly higher Ad DNA and lower luciferase production in the spleen. In contrast, the fiber-modified Ad vector would be taken up more in the liver via receptor-mediated uptake and nonspecific uptake, resulting in significantly lower Ad DNA in the other organs, especially the spleen. Even though the amount of AdK7-L2 uptake in the spleen, heart, lung, and kidney was less than that of Ad-L2 uptake, the amount of receptor-mediated uptake in these organs would be similar between Ad-L2 and AdK7-L2, suggesting that these vectors showed similar levels of luciferase production in the organs other than the liver.

The initiation of inflammatory innate immune responses occurs after the systemic administration of Ad vectors to animals and humans, and this toxicity limits the utility of Ad vectors for gene therapy. Increased cytokine/chemokine production after the injection of Ad vectors has been reported to be due to the introduction of input Ad vectors to Kupffer cells in the liver and DC (15, 17, 43–46). Detailed analysis of the organs responsible for the expression of cytokines, chemokines, and IFNs by RT-PCR suggests that their production can mainly be attributed to spleen cells (especially splenic conventional DC), not liver cells (Figs. 3 and 4), which is consistent with the recent report of Bart et al. (47). Therefore, interference with spleen distribution of the Ad vector should provide a useful method for safer gene therapy.

TLRs, which are crucial to the recognition of pathogen-associated molecular patterns, are expressed on various types of immune cells including macrophages, DC, B cells, splenic types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells (48). For example, HSV and CMV (dsDNA virus) activate inflammatory cytokines and type I IFN secretion by the stimulation of TLR9 (49–53). The innate immune receptor to the Ad has not yet been identified. It has not even been determined whether TLRs are involved in Ad-mediated innate immune response in vivo, although it has been reported that TLR signals are not involved in the DC maturation induced by the Ad vector (46). As shown in Fig. 3*B*, cytokine production against the Ad vector occurred mainly in conventional DC. It is noted that the TLR9-mediated innate immunity responses to DNA virus are cell type-specific and limited to plasmacytoid DC (50). The unidentified sensor receptor(s) for double-stranded Ad DNA or Ad capsid protein in conventional DC might play a critical role in the expression of inflammatory cytokines/chemokines and type I IFN. Although we have previously reported that large amounts of conventional Ad vector accumulate in nonparenchymal cells, including Kupffer cells and liver sinusoidal (endothelial) cells (22, 54), the expression of mRNA of cytokines, chemokines, and IFNs in the liver was weak after administration of the Ad vector (Fig. 3*B*). A lack of putative sensor receptor(s) against Ad or the inability of sensor receptor(s) to recognize Ad due to the specific cellular disposition of Ad in Kupffer cells might result in a reduced production of cytokines/chemokines/IFNs in the liver.

Another interesting finding is that the fiber-modified Ad vector containing the K7 peptide showed almost background levels of AST activity, which reflects liver toxicity (Fig. 2*B*). Histological analysis supported this finding (Fig. 2*C*). Because the K7-modified Ad vector showed higher transgene activity and a higher accumulation of viral DNA into the liver (Fig. 1), the transduction and distribution of the vector into the liver did not participate in liver toxicity. The cytokines/chemokines play a major causative role in liver damage associated with systemic Ad infusion as well as in the induction of an antiviral immune response (2). Ad-induced cytokines/chemokines recruit immune effector cells (neutrophils, monocyte/macrophages, and NK cells) to Ad-transduced cells (mainly liver), resulting in acute hepatic toxicity. Shayakhmetov et al. (35) have reported that hepatocytes and Kupffer cells trigger IL-1 transcription in liver tissue after i.v. administration of Ad vectors and that interference of IL-1-signaling reduces liver toxicity. We speculated that IL-6 could be the main mediator for hepatic toxicity because IL-6 is one of the main cytokines in the early stages of inflammation, IL-6 production by the fiber-modified Ad vector was much reduced (approximately a quarter) compared with that by the conventional Ad vector, and all of the cytokines/chemokines/IFNs we examined (including IL-6) were mainly produced by the spleen, not the liver. Treatment of the anti-IL-6R Ab decreased liver toxicity (Fig. 5), suggesting that IL-6 plays at least some role in liver toxicity induced by systemic injection of the Ad vector. Because the AST levels were only partially reduced by the treatment with the anti-IL-6R Ab, another mechanism such as IL-1 signaling, rapid Kupffer cell death (55, 56), activation of the liver endothelium (55), or other factors might be involved in the liver toxicity. Nevertheless, it is attractive that the K7-modified Ad vector did not show liver toxicity despite the higher transduction efficiency and higher accumulation of the vector into the liver (probably Kupffer cells).

Our present study provides new insight into the cellular biological mechanism related to the innate immune response and liver toxicity against the systemically administered Ad vector. Modification of vector tropism should contribute to safe gene therapy procedures.

Acknowledgments

We thank Misae Nishijima and Haiying Huang for their technical assistance.

Disclosures

The authors have no financial conflict of interest.

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〔医薬品研究
Pharm. Regul. Sci.
38(1).14～23(2007)〕

Biotechnology（品質）に関するガイドラインの動向について**

早川堯夫*

1. はじめに

本稿では，Table 1に示すようにバイオ医薬品新規課題に関する検討経過，横浜会議での議論の経過，更に品質確保と製造方法問題で考慮しておくべきことについて説明します．

2. バイオ医薬品新規課題に関する検討経過（Table 2）

バイオ医薬品の新規課題候補についての議論は，2004年11月の横浜会議で開始され，2005年5月のブリュッセル会議から本格的な議論の開始を計画していました．しかし米国側が提出案の未完成を根拠に延期を主張したため，結局，11月のシカゴ会議でIWGが開かれました．

EU，EFPIAからは製造方法，EFPIA，MHLWからはモノクロナル抗体（Monoclonal Antibodies: MoAb），更にMHLWからバイオ後続品が提案されましたが，米国側からは提案がありませんでした．日本側は，製法は各極の承認制度や方針が影響する課題との理由で保留しましたが，FDAとPhRMAが製法を支持したため，日本以外の4団体が支持したバイオ製法関連課題に関する各国合意のコンセプトペーパーの作成が可能かどうかを検討し，次回の横浜会議で結果を報告することがステアリングコミッティ（SC）で決定されました．

SCの結果を受け，EFPIAがラポータとなり，電話会議あるいはメールによってコンセプトペーパーの作成及び改訂作業を横浜会議を目指し精力的に実施しました．

ところが会議直前の2006年5月中旬になって，FDAが突然，バイオ・化成品原薬製造の双方をカバーするガイドラインの必要性とこれに関するコンセプトペーパーを作成すべきこと，バイオ単独のEWGの立ち上げには同意できないこと，統一ガイドラインは各種原薬製造にQuality by Designの概念を導入すること，Q8グループとの将来の共同作業などを骨子とする新規提案（Table 3）を行ってきました．

関係者間で相談しましたが，そのような唐突な提案にはもちろん急には対応できないということで，ラポータは当初の予定に従いコンセプトペーパー作成の詰めの作業を行ない，横浜で議論することになりました．5月末に示された最終案はMHLWからのコメントを完全に反映したものとなっていましたので，日欧対米の構図となりました．

3. 横浜会議での議論の経過

3.1 検討経過

このような状況を踏まえ，2006年6月の横浜会議の冒頭で，SCから緊急にバイオIWGで協議し報告して欲しいとの要請がありIWGが開かれました．このIWGでの議論の内容と主な意見は，要約すると次のようなものでした：1）コンセプトペーパーはほぼ最終局面に来ている，2）FDAのNCEガイドラインを含めたいとの要望に対する考慮の余地があるか検討してみてはどうか，3）Q8の概念の大半は，既にバイオ医薬品では当然のこととして実施されている，4）同じ概念が異なる表現で述べられていることに関しては，これを示す必要がある．

上記2）については更に，バイオとNCEを組み合わせた場合にどうなるか（Fig. 1）といった点について検討されました．

バイオ/NCE統一ガイドラインについては，一部の重複を回避できるというメリットもありますが，

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** 当協会主催の第14回ICH即時報告会（平成18年7月26日）における講演による．

Table 1 ICH横浜会議

- バイオ医薬品新規課題に関する検討経過
- 横浜会議での議論の経過
- 品質確保と製造方法問題で考慮しておくべきこと
  - ➢ 医薬品の品質確保の目的と手段の識別（バイオでは識別）
  - ➢ 医薬品品質確保方策全体の中で製造方法が果たす役割と位置づけの再確認（バイオでは確認済み）
  - ➢ 同一線上にない各極の承認制度や方針を認識
  - ➢ CTDM3，QOS，承認書と製造方法の取扱いの整理
  - ➢ 開発時，承認時，市販後の各段階における製造方法問題のとらえ方

一方，これまでのバイオの準備作業が活かされない，Q8Rの進捗状況から今後の見通しが不透明である，議論が複雑化し，作業に時間がかかることなどを含めて，さまざまなデメリットが考えられました(Table 4)．

そこで，IWGの結論としては，シカゴ会議での計画に基づき寄せられたコメントについて検討を行いながら，コンセプトペーパーの作成作業を継続し，各種オプションについては更に検討を続けることとなりました．仮にNCEを含むとしても，現在のIWGで作業を継続してコンセプトペーパーを完成し，引き続きガイドライン作成作業を早期に開始するとの結論になりました．

このような考えをSCに報告したところ，FDA，PhRMAからバイオ単独での作業には異論があるとの意見が出ましたが，日欧はIWGを支持するとの意見でした．結局，その際のSCの結論は，横浜会議ではNCEの専門家が不在であるため，NCE部分はペンディング状態で，現在のコンセプトペーパーの合意形成を目指す方向で了承するということでした．

そこで，IWGはコンセプトペーパーの作成作業を続行し，各極IWGが合意に達する段階に至りました．そして念のため各極にいったん持ち帰っての検討・確認することとなりました．

翌日，FDAから，将来バイオとNCEの原薬製造ガイドラインを統合する機会を考慮するとの文言を入れて欲しいとの提案があり，脚注として付記することとし，以上でIWGでの6者合意のコンセプトペーパーが完成しました．

引き続き，ガイドラインの作成作業の日程やビジネスプラン等を完成し，更にガイドライン骨子の検討，各項に含むべき主要事項に関する検討を進めました．

3.2 調和ガイドラインについての検討

検討の結果，調和ガイドラインのタイトルは「バイオ医薬品/生物起源由来医薬品原薬の製造」とし，全体のコンセプトは，製品の品質と恒常性を確保する方策全体の一部としての製造方法に関する科学的，技術的原則の調和ということになりました．

対象範囲は，CTD-QのS 2.2から2.6の部分で，

Table 2 バイオ医薬品新規課題に関する検討経過

- 2004年11月（横浜）：コンパラビリティGLの終了を受けて，新規バイオ課題候補について議論
- 2005年5月（ブリュッセル）：本格的議論開始を計画；米国側が提出案の未完成を根拠に延期を主張
- 2005年11月（シカゴIWG：非公式専門家会議）：EU/EFPIAから製造方法；EFPIA/MHLWからMoAb；MHLWからバイオ後続品を提案；米側は提案なし．日本側は製法は各極の承認制度や方針が影響する課題ということで保留したが，FDA/PhRMAが製法を支持．
- 2005年11月（シカゴSC）：4団体が支持したバイオ製法関連課題に関する各国合意のコンセプトペーパの作成が可能かを検討し，横浜会議SCに結果を報告すること．
- 2005年12月以降，EFPIAがラポータとなり，数回の電話会議とメールによるコンセプトペーパの作成，改訂作業を実施．
- 2006年5月中旬：FDAが突然，①バイオ・化成品原薬製造の双方をカバーするGLの必要性とこれに関するコンセプトペーパを作成すべきこと，②横浜でのバイオ単独のEWGの立ち上げには同意できないこと，③統一GLは各種原薬製造にQbDの概念を導入すること，④Q8グループとの将来の共同作業などを骨子とする新規提案（Table3 参照）
- 2006年5月末：ラポータによるコンセプトペーパ最終案はMHLWからのコメントを完全に反映，日欧*vs*米の構図

Table 3 FDA: Combined Concept Paper DS Manufacture

- No support for a biotech-specific guideline on DS manufacture
- FDA proposes:
  - Comprehensive guideline for drug substance manufacturing processes
- Scope:
  - New chemical entities AND biotech products
  - No re-examination of issues already addressed in Q8 or Q7A
  - Complement guideline with an annex to address biotech topics not covered in the general DS guideline.
- Objective:
  - Implementation of QbD principles into a variety of drug substance manufacturing processes (synthesis, fermentation, cell culture, downstream purification, etc.)
  - High level guideline relating to the CTD-Q S2.2-S2.6 sections
- Contents:
  - Scientific principles relating to the manufacturing process as one part of a total control strategy designed to ensure quality and consistency of Drug Substances with regard to Drug Product quality.
- Next steps:
  - Get input from Q8 EWG on the idea of developing a combined DS concept paper.
  - Ensure Steering Committee support for Q5 IWG exploring the development of a combined DS concept paper.
  - Biotech discussion group will meet in Yokohama to:
    - discuss the positions of the six Parties with regard to the scope of the Concept Paper
    - develop a concept paper proposing a more comprehensive DS guideline
    - develop an outline of principles topics that should be included in the proposed guideline.
    - identify rapporteur for the new concept paper will need to be identified
    - The establishment of an EWG for biotech DS in Yokohama is premature at this time.
  - Provide concept paper to the Q8 EWG to get additions/revisions relevant to non-protein drug substances.
  - Approval of CP/Bus. case & EWG by ICH SC (telecom) in summer 06
  - DS EWG to meet in Chicago in October 06

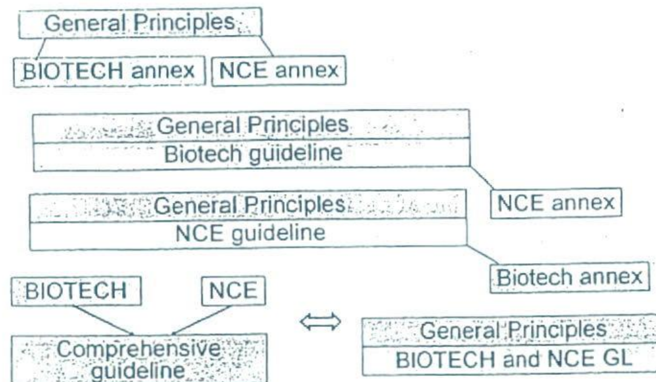

Fig. 1 What would the guideline look like?

対象物はQ6Bの定義にあるバイオ医薬品/生物起源由来医薬品の原薬ですが，原則としてその他の医薬品原薬にも適用できる場合もあるということです．また，本ガイドラインの作成作業とは別に，将来，バイオ医薬品，化成品原薬の製法に関するガイドラインを統合する機会もあるかもしれないことも付言しました．

ガイドラインの目標の一つ目は，原薬の品質とその恒常性の確保を保証できる製法とするための科学的な考え方の概略とすること，二つ目は，一定の品

Table 4　バイオ／NCE 統一ガイドラインのメリットとデメリット

- メリット
  - 統一ガイドライン
  - 一部の重複を回避
- デメリット
  - バイオでの1年間の準備作業が活かされない
  - バイオはさらに待機を余儀なくされる
  - Q8R の進捗状況から予測すると，作業開始は少なくとも明年春以降となる．今後の見通しが不透明
  - 議論の複雑化，多くの調整が必要，作業に時間がかかる
  - 大勢の EWG が必要
  - 各極のサポートが得られるか？
  - 各種製品に特有の事項のカバーに工夫を要する

質の製品を確実に生産できる優れた製造方法とする（あるいは製造方法である）ためにはどのような目標を持ち，どのような検討が必要かを明確にすること，三つ目は，申請資料が製造方法に関する情報とその妥当性に関して適切であることを推進すること，四つ目は，承認審査の迅速化，五つ目は，製法変更に関して，規制がより柔軟に対応できるように製法及び製品に関する知見をいかに示せばよいかの方策を確立することです．

タイムテーブルは，Table 5 に示すように 2006 年6月に立ち上げを行い，1年9箇月後の 2008 年3月に Step 2 に到達し，更にその1年後に Step 4 に到達する予定としました．

3.3　IWG と SC の結論

IWG としては，コンセプトペーパーとビジネスケースに関して6者合意に達した，ということで，本課題及び提案した作業日程が SC で是認されるこ

Table 5　Planning - Timelines

Step	Responsible	Timeline
Approval of the topic / Rapporteur / EWG defined	SC	Jun-06
Generation of Draft Outline of Contents (Meeting)	Rapporteur	Jun-06
Review of Draft Outline of Contents	Experts	Jun-06
Integration of comments and distribution of finaldrat	Rapporteur	Jun-06
EWG teleconference(s) to agree on Table of Contents	EWG	Jul-06
Generation of Draft 0 and distribution to EWG	Rapporteur	Aug-06
Discussion of Draft 0, generation of Draft 1 (Meeting)	EWG	Oct-06
Review of Draft 1	Experts	Jan-07
Reconciliation of Comments, generation of draft 2	Rapporteur	Feb-07
Discussion of Draft 2, generation of draft 3 (Meeting)	EWG	End Mar-07
Review of Draft 3 by individual parties	Experts	Jul-07
Reconciliation of Comments, generation of draft 4	Rapporteur	Sep-07
Discussion of Draft 4, generation of draft 5 (Meeting)	EWG	Nov-07
Review of Draft 5	EWG	Jan-08
Implementation of comments received	Rapporteur	Feb-08
Generation of Draft 6 (Step 2 Signoff, Meeting)	EWG	Beq Mar-08
Translation (Japan) / Internal consultation / discussion	Regulators	Jun-08
Release for public consultation	Regulators	Jul-08
Public comments received	Public	End Dec-08
Public comments integrated	Rapporteur	Feb-09
Discussion of Step 3 document (Meeting)	EWG	Mar-09
Step 4 Sign-off	Regulators	Mar-09

と，ラポータの指名，本課題のトピックコードの命名についてSCに対して要望しました．

ところがSCでは，PhRMAとFDAから，①NCEやQ8，Q9，Q10ガイドラインと考え方が同じか否かが不明瞭である，いい換えれば，Q全体として同一のかさのもとでの考え方の整合性が必要である，②ガイドライン作成作業の効率や効果に問題がある，③Quality by Designに通底する目標を明確に表現する必要があるなどといった理由により，現行案でのコンセプトペーパー等の是認はできず，修正が必要であるとの意見が出ました．

ラポータは，Quality by Designの解釈は多様であり，これをコンセプトペーパーに明確に反映することは困難であると反論しました．

それに対しヨーロッパや日本のSCメンバーはIWG案を支持するとの意見でしたが，米国側が譲らず，結局6者の合意には至りませんでした．これを踏まえ，再度IWGで議論することとなりました．

3.4　IWGでの再度の議論

IWGでの議論に欧米のQ8グループが来て，Quality by Designやデザインスペース等のコンセプトは極めて優れた上位概念として目指すべきものであることを主張するとともに，一部SCの意向に合わせるポーズをとることを薦めました．

IWG内ではQ8，Q9，Q10の概念，一般原則を考慮する旨の記述をいかにコンセプトペーパーに取り込むか議論しましたが，この議論は必ずしも科学的必然性による動機からのものではなく，妥協点を見出そうとする側面が強いものでした．したがって，実際にはQ8やQ10という言葉をどこにどう入れるのかといった議論に終始したということでした．そのような中でEUとEFPIAも次第にFDAやPhRMAに近いポジション取りに変わっていきました．

しかし，MHLWとしてはIWGで科学的に合意できていたコンセプトペーパーを再改訂することは不適切と判断しました．その理由として，①経緯が不当であること，②米国SCの意見の根拠が合理的でないこと，③原薬製法に関連するQ8，(Q9)，Q10の概念は未だ明瞭ではなく，その理解や解釈が多様であること，今後これらが共通のものとして確立する可能性があるとしてもその時期は不確定であること，④これらを上位概念とすることが妥当とは考えられないこと，⑤ガイドライン作成が効率的でないこと，⑥国際調和の名の下で特定地域のあるポリシーをあまねく他の地域に強制しようとすることは不当でアンフェアではないかということ，⑦欧米主導で進められる可能性があるガイドラインが果たして我が国にとって利益があるのか疑問である，などといったことが挙げられます．

結局，既にIWG 6者で合意された事項を超えて新たな合意形成が得られなかったことをラポータはSCに報告しました．

4．品質確保と製造方法問題で考慮しておくべき点（Table 6）

バイオ医薬品の原薬の製法に関するガイドラインの作成はブレーキがかかった状態となりましたが，その後のSCでバイオとNCE統一化問題に関するブレーンストーミングセッションを次回のシカゴ会議で開催することとなったと聞きました．これは必ずしも好ましい展開であるとは思えません．しかし，そのセッションに備えて品質確保と製造方法問題に関し考慮しておくべきことを整理し，理論武装しておく必要があります．その際，さまざまな次元の異なる切り口から考える必要があると思い，Table 6に示すように整理してみました．

4.1　医薬品の品質確保の目的と手段の識別

まず，医薬品の品質確保の目的と手段をきちんと識別しておく必要があると思います．

品質確保・保証・管理の目的は，「最終製品の有効性・安全性確保」にあります．このことが最も大事なコンセプトで，最上位概念として位置づけられるべきものです．

一方，品質確保に関連する様々な方策はいずれも

Table 6　品質確保と製造方法問題で考慮しておくべき点

- 医薬品の品質確保の目的と手段の識別（バイオでは識別できている：Q5E）
- 医薬品品質確保方策全体の中で製造方法が果たす役割と位置づけの再確認（バイオでは確認済み：Q5シリーズ）
- 同一線上にない各極の承認制度や方針を認識
- CTDM3，QOS，承認書における製造方法の取扱いの整理
- 開発時，承認時，市販後の各段階における製造方法問題のとらえ方