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原　著

C型肝炎ウイルスRNAの遺伝子検査法のための第一次国内標準品の作製

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ESTABLISHMENT OF THE FIRST NATIONAL STANDARD FOR NUCLEIC ACID AMPLIFICATION TECHNOLOGY ASSAY FOR HCV RNA

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The First WHO International Standard for HCV RNA for Nucleic Acid Amplification Technology (NAT) Assay (96/790) was established in 1997. The aim of our collaborative study was the establishment of the Japanese National Standard for HCV RNA calibrated against the WHO International Standard. The candidate materials were evaluated in the following two steps. First, titers of two HCV positive plasma (119 and 122) diluted in cryosupernatant were evaluated, and plasma 122 was chosen as the source plasma for the candidate for the national standard. Then, candidate 122 was prepared by diluting the source plasma to approximately 10^5 international units (IU)/m*l* in cryosupernatant. The relative potency of the candidate was measured against the International Standard by the end-point method. Seven laboratories from three countries participated in the collaborative study. Four laboratories used the Roche Amplicor assay (Version 1) and 3 laboratories used in-house PCR methods. There was reasonable agreement among the mean estimates from the laboratories. The overall mean potency of the candidate relative to the International Standard was $10^{5.00}$ ($10^{4.80}$～$10^{5.20}$) IU/m*l*. The sample was accepted as the first Japanese national standard and assigned a titer of 100,000 IU/m*l*. Each vial of the National Standard contains 0.5 m*l* of HCV plasma (genotype 1b) diluted in cryosupernatant and should be stored at－80℃.

Key words : HCV, The WHO International Standard, National Standard, Nucleic acid technology (NAT) assay, Blood safety

1. はじめに

供血者のC型肝炎ウイルス（HCV）に対する抗体スクリーニングを実施したにもかかわらずヨーロッパとアメリカ合衆国では血漿分画製剤によるHCVの感染が報告された．これは，HCVに感染してから抗体が検出されるまでのウインドウ期の血漿が原料血漿に混入していたためと考えられた[1][2]．そこで，血液製剤のより一層のウイルス学的安全性の確保を目的としてヨーロッパでは1999年7月1日から原料血漿プールでHCV-RNAの核酸増幅検査（NAT）を実施することになった．すでにイギリスをはじめオランダ，ドイツ，イタリア，アメリカ合衆国の各国では標準品やランコントロールを作製しており，NATを実施する施設で使用されていたが，HCV-RNA量がコピー数やgenome equivalent等まちまちの単位で表示されていたので，標準品のHCV-RNA量やNAT法の感度を相互に比較することが出来なかった．イギリスのNIBSCによってHCV-RNAの国際標準品作製のための国際共同研究が組織され，1997年10月にWHO国際標準品（96/790）が制定され，国際単位を用いて各国参照品の力価を比較することが可能になった[3][4]．わが国においては厚生省告示第427号によって，平成13年3月1日から製造され，又は輸入される血液製剤の原料血漿についてB型肝炎ウイルスDNA，C型肝炎ウイルスRNA及びヒト免疫不全ウイルスRNAに対するNATを実施しなければならないことに改められた．実際にはそれ以前に日本赤十字社の献血血液とすべての血漿分画製剤製造所の原料血漿プールについてHCV-RNAのNATが実施された．しかし，施設ごとにNAT法が異なり，自家標準品やキットの標準品の表示単位が統一されていなかったので，それぞれの施設での感度や精度を比較・評価することができなかった．国際単位で表示された広く認められた標準品を用いて感度や精度を測定することにより，施設間の比較や評価が可能になると考えられた．一方，国際標準品

はその配布数も限られており，国際標準品に対して較正された我が国独自の国内標準品の作製が望まれていた．そこで，血漿分画製剤の安全性確保対策の検討小委員会（以下，小委員会と略）はHCV-RNA量を国際単位で表示した国内標準品を作製するための共同研究を組織し，第一次HCV-RNA国内標準品を作製したので報告する．国際標準品はgenotype 1であるが，国内標準品は我が国で最も頻度の高いgenotype 1bとした．現在，さまざまなウイルスについて臨床や研究の場でNATが実施されているが，国内標準品として定められたものはまだない．その意味で，本標準品は我が国で初めて作製されたウイルスのNATのための国内標準品でもある．

2. 材料および方法

1）国内標準品候補の原料血漿の選択

日本赤十字社より供与されたHBs抗原，抗HIV-1/2抗体，HBV-DNA，HIV-RNAのすべてが陰性で，HCV陽性の血漿の中から日本で最も高頻度に見られるgenotype 1bの2つの血漿（119と122）を標準品の原料候補とした．各原料血漿の一部を脱クリオプール血漿で約10^5国際単位（IU）/m*l*に希釈して−80℃で凍結・保存した試料を調製し，HCV-RNA国際標準品とともに参加施設に配布した．各施設は測定ごとに新しいバイアルの候補品を脱クリオ血漿で希釈して10倍希釈系列（10^{-1}から10^{-7}）を調製することとし，日を替えて2回定性的な方法でエンドポイントの測定を実施した．一重測定を原則としたが，日常的に二重測定を実施している場合は二重測定した（第1回測定）．このとき使用した国際標準品は小分けして−80℃に凍結保存して第2回測定に用いた．

2）HCV-RNA国内標準品候補の作製と評価

1）で選択した血漿122（PHA力価2^{14}，RNA量2〜3×10^6IU/m*l*，容量185m*l*）をあらためて約10^5IU/m*l*に脱クリオ血漿で希釈，0.5m*l*ずつガラス瓶に分注し−80℃で凍結して，HCV-RNA国内参照品候補122（候補品）とし，参加施設に送付した．各施設は初回は10倍希釈系列で予備的なエンドポイントを測定し，より正確なエンドポイントの値を得るために2回目以降はそのエンドポイントをはさんで7段階の$10^{0.5}$希釈系列を測定ごとに調製し，日を替えて4回測定を実施した（第2回測定）．参加施設から返送された結果を集計して，HCV-RNA国内標準品候補のWHO国際標準品に対する力価を推定した．

3）参加施設と測定方法

日常的にHCV-NATを実施している9施設（国内6施設，米国2施設，ヨーロッパ1施設）に候補品を配布し，7施設（国内5施設，米国1施設，ヨーロッパ1施設）から試験結果が返送された．核酸の抽出と増幅の方法は各施設の任意の方法で実施した．

4）測定値の分析

候補品，国際標準品についてそれぞれのエンドポイント濃度の対数値の平均を求め，その比を国際標準品に対する候補品の対数相対力価とする．施設ごとに国際標準品に対する候補品の対数相対力価とその95% 信頼区間を推定した．7施設から得られた対数相対力価の加重平均を求めて候補品の対数相対力価を推定した．対数相対力価の真数は国際標準品に対する候補品の相対力価を現すので，真数の値を国際標準品の力価に乗じて候補品の力価を推定した．

3. 結　果

1）参加施設が実施した測定方法

血漿分画製剤製造所5施設（国内3，海外2），公的機関1施設，その他1施設の合計7施設から結果が返送された．Table 1に参加施設を表すコード番号，抽出法，検出法を示す．4施設がアンプリコアHCV(Ver. 1)変法，2施設が自家法のnested PCR法，1施設が自家法のsingle PCR法を用いて測定した．反応当たりの試料の量は40〜400μ*l*の血漿に相当した．

2）原料血漿の選択

国内標準品は様々なNAT法に使用されるので，候補品にふさわしい原料を選択する目的で，第一回測定では2つのHCV陽性血漿119と122を希釈した試料を配布して測定した．大きな相違がなかったので，より多くの標準品の作製が可能なように容量の大きい血漿122を候補品の原料として選択した．血漿122のHCVコア領域の塩基

Table 1 Assays used in the collaborative study.

Laboratory	Assay	Extraction[a]	Eq. Vol. Amplified[b]
1	Amplicor	R&D	100
2	In-house single PCR	In-house NaI	40
3	Amplicor	Amplicor	50
4	Amplicor	R&D	100
5	Amplicor	QIAamp	400
6	In-house nested PCR	R&D	100
7	In-house nested PCR	R&D	100

a) R&D : Smi-test EX-R&D (Nippon Genetics Co. Ltd.)
Amplicor : Amplicor HCV version 1 (Roche)
QIAamp : QIAamp DNA Blood Mini Kit (QIAGEN)
b) Eq. Vol. Amplified : the equivalent volume of sample that was amplified in an assay

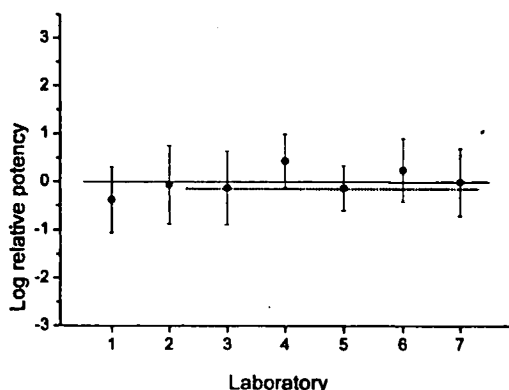

Fig. 1 Log relative potency of candidate 122 to the international standard (96/790). The laboratory code number and assay methods are explained in Table 1. The solid line indicates the mean log relative potency calculated from all data, −0.001 (−0.204-+0.201). The dotted line indicates the mean log relative potency calculated from the data excluding those of the laboratories 1 and 2, +0.066 (−0.161-+0.292).

Table 2 Estimated log potency of candidate 122 calibrated against the international standard (96/790). Overall (a) = the overall mean log potency calculated from all laboratories. Overall (b) = the overall mean log potency calculated from data excluding those of laboratories 1 and 2.

log10IU/ml

Laboratory	Mean	Minimum	Maximum
1	4.63	3.94	5.31
2	4.94	4.12	5.75
3	4.88	4.11	5.66
4	5.44	4.88	6.00
5	4.88	4.41	5.34
6	5.25	4.59	5.91
7	5.00	4.29	5.71
Overall (a)	5.00	4.80	5.20
Overall (b)	5.07	4.84	5.29

配列を決定して genotype 1b であることを確認した.

3) 候補品 122 の国際標準品 (96/790) に対する力価の推定

あらためて候補品を送付し, 7 施設において $10^{0.5}$ 稀釈系列で測定した (第 2 回測定). 5 施設で独立の 4 回の測定, 2 施設で各 2 回繰り返し測定を独立に 4 回行った. エンドポイント法により国際標準品に対する候補品の対数相対力価を求めた. なお, エンドポイントが最大希釈と同等となった場合は最大希釈をエンドポイントとした. また, 不連続な陽性結果を含む場合は希釈率の高いほうをエンドポイントとした. 施設毎に候補品の国際標準品に対する対数相対力価とその 95% 信頼区間を求め, 全施設の測定結果を用いて候補品の国際標準品に対する対数相対力価を推定した. Fig. 1 に示すように全施設の結果は誤差の範囲で一致し, 国際標準品に対する候補品の対数相対力価の平均は $\log 10^{-0.001}$ であった. WHO 国際標準品 (96/790) の力価は 10^{5}IU/ml であるから, 候補品の力価は $10^{5.00}$ ($10^{4.80 \sim 5.20}$) IU/ml, 即ち 100,000IU/ml

と推定された（Table 2）.

参加7施設中，施設1では測定4回中3回でエンドポイントが最大希釈と同等となった．また施設2では不連続な陽性結果が多く，測定結果のばらつきが大きかった．そこで，この2施設を除く5施設の測定結果を用いて分析した結果，5施設の結果は誤差の範囲で一致し，国際標準品に対する候補品の対数相対力価の平均は $\log 10^{0.066}$ であった（Fig. 1）．よって，候補品の力価は $10^{5.07}$（$10^{4.84\sim5.29}$）IU/m*l*，即ち116,300IU/m*l* と推定され，全施設の結果を用いた分析結果と有意な相違は認められなかった（Table 2）．最尤法で本研究の測定値を分析すると候補品の推定力価は $10^{5.07}$（$10^{4.86\sim5.30}$）IU/m*l* となり，2つの分析法による推定値はよく一致した.

以上の結果から，候補品122の国際標準品に対する力価は $10^{5.00}$IU/m*l* と推定され，力価100,000 IU/m*l* の国内標準品として1999年12月に小委員会で承認された.

4. 考　察

一般に個々の施設で国際標準品に対する2次標準品を作製すると新たな誤差が生じるので，異なる2次標準品を用いて測定した結果を相互に比較するのは困難である．HCV-RNA NAT試験において異なる施設間での測定値の比較や施設毎の検出感度の管理を実施するためには性状が詳しく調べてある広く認められた共通の標準品が必須である．本共同研究によってわが国で初めて，国際単位表示されたHCV-RNAの国内標準品が制定された．候補品の95%信頼区間は力価 $10^{5.00}$IU/m*l* に対して $10^{4.80\sim5.20}$IU/m*l* であった．また参加施設のなかの力価の最大は $10^{5.44}$IU/m*l*（施設4），最小は $10^{4.63}$IU/m*l*（施設1）で $10^{0.81}$ 倍の相違であった（Table 2）．これらの値はエンドポイントの測定を $10^{0.5}$ 倍希釈系列で実施したことを考慮すると十分に小さいといえる．これは本共同研究の参加施設を日常的にHCV-NATを実施している信頼性の高い施設に限ったためと考えられる．国内標準品は分与される予定であるので，血液製剤の安全性確保のためのNAT試験法や診断薬の評価，臨床検査センターにおけるHCV-RNA検査の評価に広く用いられるようになれば，相互の性能を容易に比較することが可能になり，試験法・検査技術の向上が期待できる．各施設で国内標準品を用いて繰り返し測定することにより有効検出限界の推定値を得ることが可能である．こうして得られた有効検出限界をもとに，たとえば95%陽性反応を得られる濃度と50%陽性反応を得られる濃度の標準品を常に測定に加えた測定結果を集積し，継続的に各試験法の感度管理の精度向上を図ることが望まれる.

5. 結　論

血漿のHCV-RNAのNATのための国内標準品を作製した．国内標準品はHCV抗体陽性のHCV genotype 1b陽性血漿を脱クリオ血漿で希釈し，0.5m*l* ずつバイアルに分注，−80℃で凍結保存したもので，その力価は100,000IU/m*l* である.

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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 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	cgacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgttttttggaaat (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	ccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgttttttggaaat (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

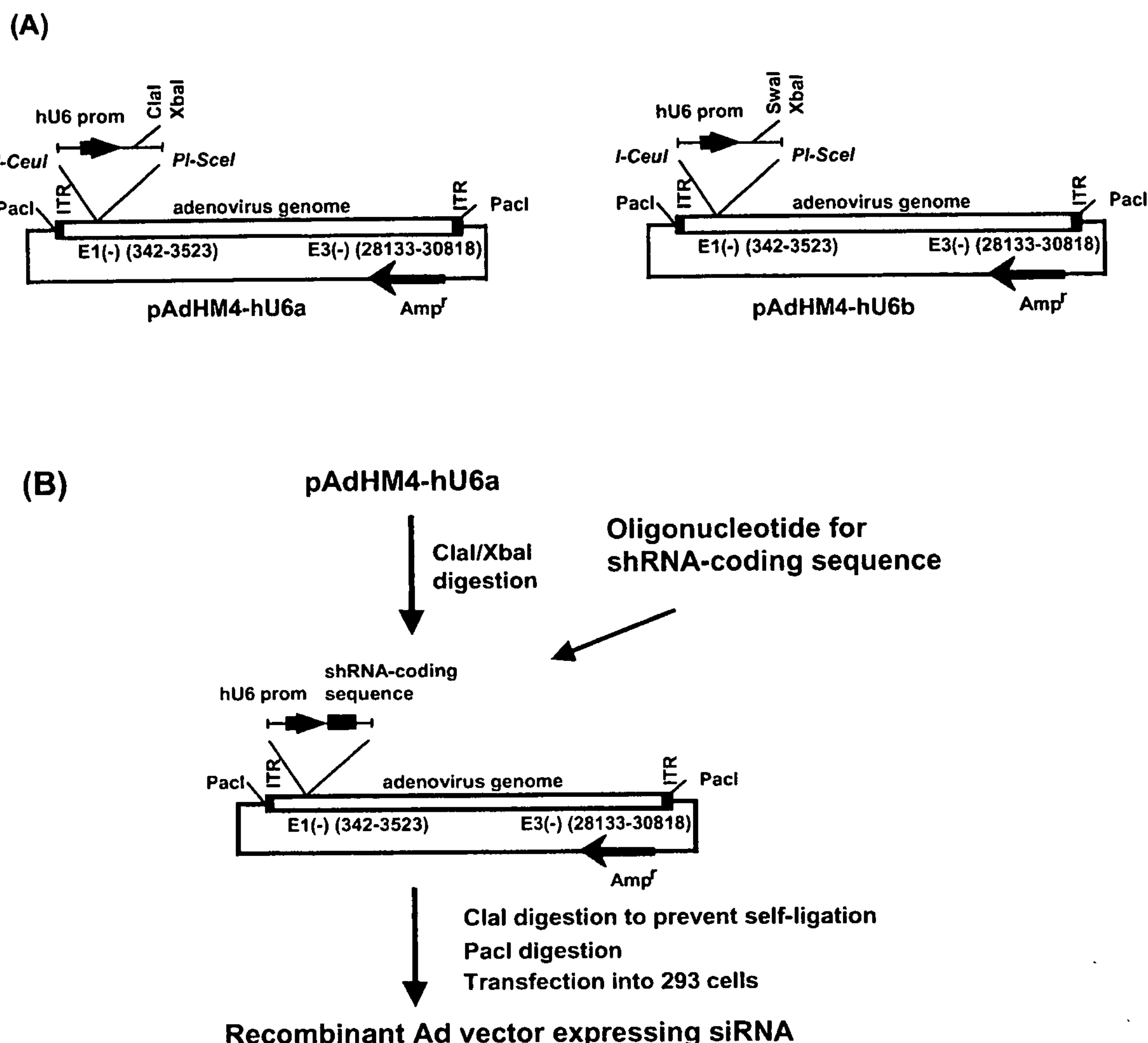

FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. (A) Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *ClaI* site at the transcription start site of the hU6 promoter sequence and an *XbaI* site downstream from the promoter sequence. pAdHM4-hU6b 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 *PacI* 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
CTT TTA G|cx
   ClaI

                ┌──▶ transcription
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)                (anti-sense)         stop
```

(C) The hU6b promoter (in this study)

```
GAA TTT|xxx
CTT AAA|xxx
   SwaI

                ┌──▶ transcription
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)                (anti-sense)         stop
```

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-

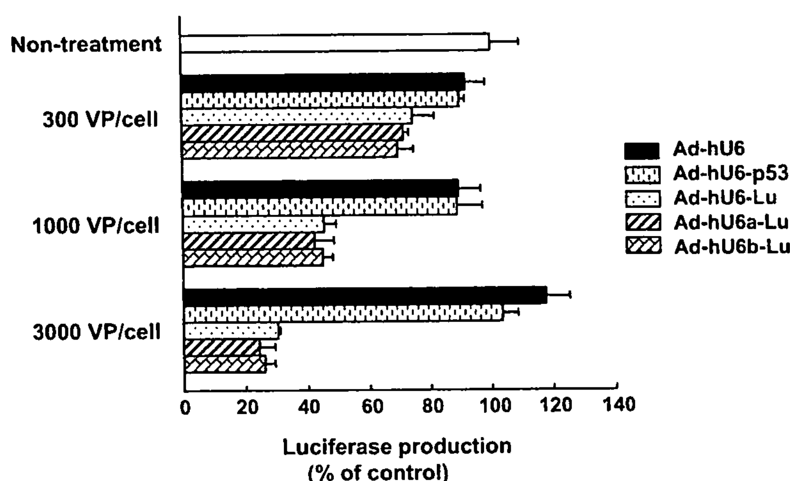

FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the 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 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

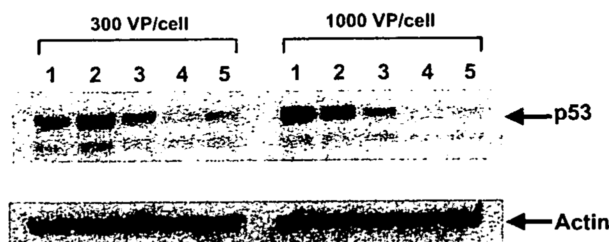

FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted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

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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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[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).

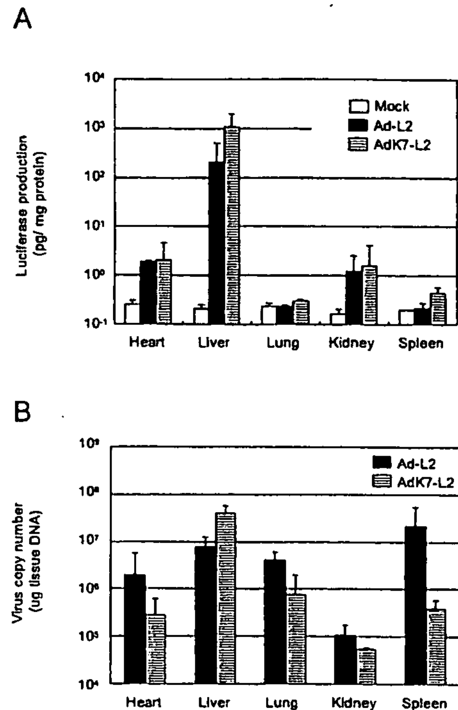

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production (*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.

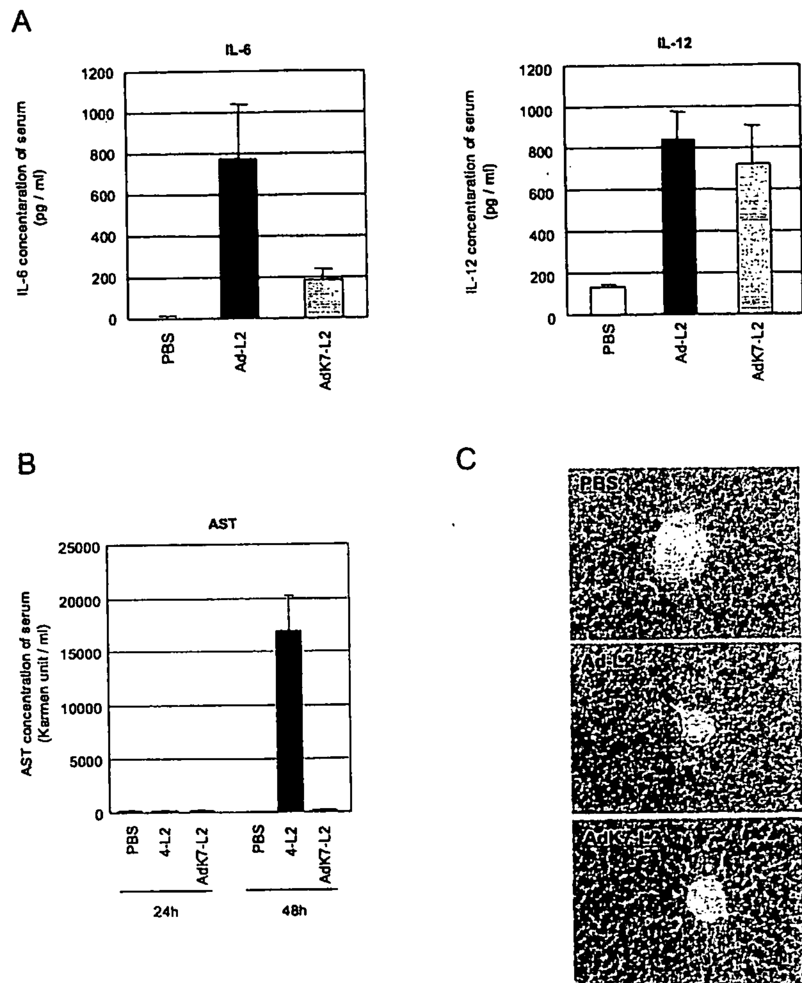

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*). 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

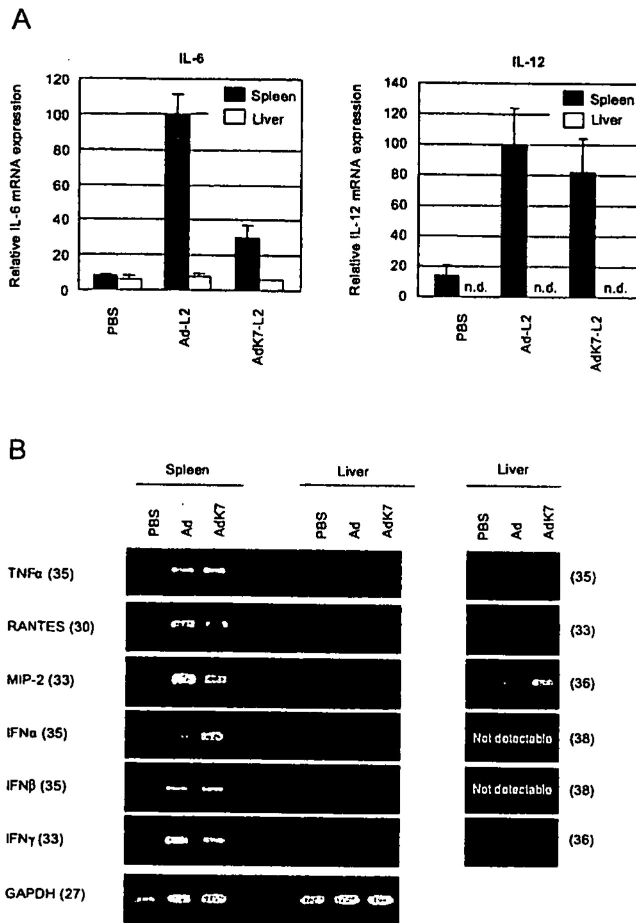

FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, 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 ± SD of four mice. Cycle number is given in parentheses.

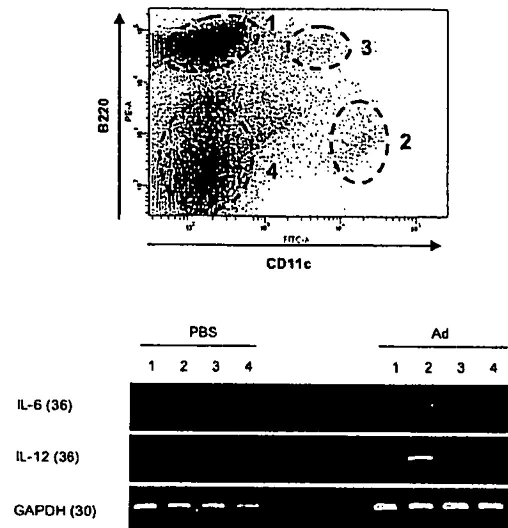

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by 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