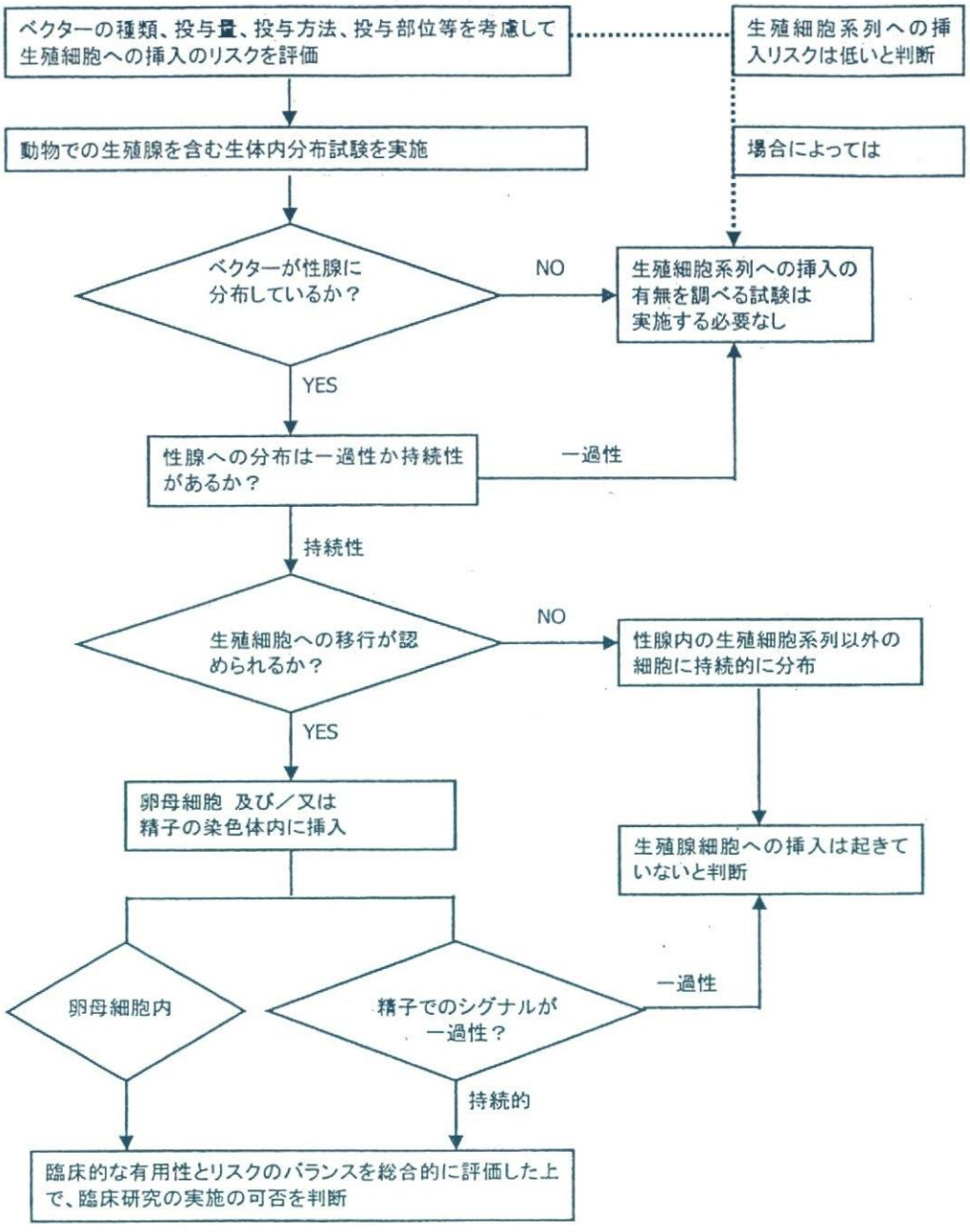

Fig. 2 遺伝子治療用医薬品の生殖細胞系列への組込みの有無を調べるための動物試験のフロー（案）

布を試験するに当たっては，定量的 PCR 等の適切な感度を持つ試験法を用いて試験をすることが求められます．

生殖腺への分布が認められた場合，そのシグナルに持続性があるかどうかを試験し，持続性が認められれば更に生殖細胞へのシグナルが認められるかどうかを明らかにします．これをフローチャートで表すと，Fig. 2 のようになります．

まずはベクターに応じてリスクを評価します．生殖細胞への挿入リスクが低いと判断された場合は，もともと生殖細胞の挿入リスクがないと考え，試験を実施する必要がない場合もあります．しかし生体内分布試験を実施してベクターが性腺に分布しないのであれば，それ以上の試験は必要ありません．こ

の評価を全ての製品について行わなければならないかについては，例えば対象が非常に高齢であったり，重篤な患者に限定される場合は，次世代への影響を考慮する必要がないことから，試験を実施する必要はないことが記載されています．

一方，ベクターが性腺に分布していた場合は，性腺の分布が一過性か持続性かを判断し，一過性であれば組み込みは起きていないと判断できますが，持続性であって，かつ生殖細胞に移行した場合は，精子の染色体内に行っているかどうかを試験し，精子でのシグナルが一過性であれば，生殖腺細胞への挿入は起きていないと判断できます．一過性でない場合，よほどの理由がない限り臨床試験は実施できないこととなります．ただし，先ほど述べたように次世代への影響が起こるような患者を対象としていない場合は判断が変わってきます．

5.2.3　患者のモニタリング（Table 11）

非臨床試験において，生殖腺内に例えば一過性のベクターシグナルが認められた場合は，臨床試験において患者の精子への伝達が無いか，特に精子の成熟サイクルの期間を越えて試験をすることが推奨されます．

6．遺伝子治療専門家会議の今後の活動予定（Table 12）

ICH 見解の策定を次の二つについても行うべきではないかと議論しました．

まず，「腫瘍溶解性ウイルス」について，2005 年のシカゴ会議でオープンワークショップを行った成果を基に ICH としての見解案，もう一つは，ウイルスやウイルスベクターの体外への放出の評価についても見解案を作成すればどうかといった意見が出ました．

更に，ICH7 での活動予定についても議論をしました．

Table 12　ICH遺伝子治療専門家会議の今後の活動予定

- ICH GTDG 今後の活動予定
  - ICH 見解の策定
    - ICH 見解「腫瘍溶解性ウイルス」
    - ICH 見解「ウイルス／ベクターの体外放出評価」
  - ICH7 での活動予定

6.1　腫瘍溶解性ウイルス

非増殖性のウイルスを用いたがん治療においては，腫瘍部位にベクターが入った部分のみがん細胞は死に，入っていない部分は生き残ります．ところが腫瘍溶解性ウイルスは，がん細胞でのみ増殖できる性質を持っており，がん細胞を破壊し，更に隣のがん細胞も破壊して増殖していくという制限増殖型ウイルスです[1]．

世界的に見ると，腫瘍溶解性ウイルスはアメリカ，カナダ，ヨーロッパで多くの製品の開発が行われています．国内では遺伝子治療としてはまだ行われていませんが，野生型あるいは弱毒化されたウイルスを用いた腫瘍溶解性ウイルスの臨床研究は行われています．名古屋大学で行われている例を Table 13 に示します．

それ以外にも動物実験の段階のステージにある製品を Table 13 に示しています．これらはほとんどが遺伝子組換えのウイルスのタイプです．

6.2　ICH 腫瘍溶解性ウイルスワークショップ（Table 14）

2005 年にシカゴにおいて，腫瘍溶解性ウイルスのワークショップが開催されました．その目的は，腫瘍溶解性ウイルスについて以下のような臨床開発に関連する問題点を整理し，意見交換を行うことです．

問題点の一つ目は，腫瘍溶解性ウイルスには，野生型，弱毒型，遺伝子組換え型がありますが，それぞれどのような設計に基づき腫瘍に特異的に作用するかについての検討です．

二つ目は，非臨床試験の有効性・安全性の確認及び至適用法・用量の検討です．

三つ目は，腫瘍選択性で，例えば腫瘍にのみ発現しているタンパク質や発現が抑えられているタンパク質をターゲットにしたり，若しくは腫瘍細胞に特異的に発現している抗原をターゲットにするといった選択性が検討されています．

四つ目は，臨床での安全性で，患者体内であれば生きたウイルスが存在し続けることも含めた安全性をどのように評価するかの検討です．

五つ目は，安全性，投与量，あるいその有効性を評価するためには適切な動物モデルを策定しなければなりませんので，モデル動物の開発についてです．

Table 13 国内開発中の腫瘍溶解性ウイルスの例（2005 年 4 月現在）

＜臨床研究段階＞

ウイルスの種類	ウイルスの名称	ウイルスの特徴	実施施設/企業名	対象疾患	実施状況	症例数	治験
変異単純ヘルペスウイルス	HF10	弱毒型の自然変異株*	名古屋大学医学部附属病院	皮膚又は皮膚に再発した乳がん	2003 年に臨床研究終了	6	—
				頭頸部がん	2004 年に臨床研究開始	5（予定）	
				進行性膵臓がん	2005 年に臨床研究終了	6	
				大腸がん，乳がん	動物実験	—	

*：天然型ウイルスのため，実施前に「遺伝子治療臨床研究指針に関する指針」に基づく厚生労働大臣の確認は不要．また，カルタヘナ法に基づく第一種使用規程承認申請も不要．

＜動物実験段階＞

ウイルスの種類	実施施設／企業名	対象疾患
遺伝子組換え単純ヘルペスウイルス	大阪府立成人病センター	平滑筋肉腫
	愛知県がんセンター	卵巣がん
	東京大学医学部	グリオーマ，前立腺がん
	慶應義塾大学医学部	脳腫瘍，前立腺がん，膀胱がん
	九州大学医学部	胆嚢がん，胆道がん
	和歌山県立医科大学	前立腺がん，腎がん，卵巣がん，乳がん
遺伝子組換えヒトアデノウイルス	岡山大学医学部/オンコリス・バイオファーマ	大腸がん，非小細胞肺がん
	東北大学医学部	膵臓がん，膀胱がん
		非小細胞肺がん
	筑波大学	胆嚢がん，胆道がん
	愛媛大学医学部	卵巣がん
		グリオーマ
	千葉県立がんセンター	肝がん，肝細胞がん
	札幌医科大学	大腸がん，肝がん
遺伝子組換えセンダイウイルス	ディナベック	大腸がん，線維肉腫
レオウイルス 3 型	大分大学医学部	膵がん，同腹膜転移

(www.nihs.go.jp/cgtp/cgtp/sec1/onclte_v/onclt-j.html)

Table 14 ICH腫瘍溶解性ウイルスワークショップ2005年シカゴ

- 目的

腫瘍溶解性ウイルスの臨床開発に関連する以下のような問題点を整理し，フロアーも含めて積極的に意見交換を行う．

- 腫瘍溶解性ウイルスの設計（野生型，弱毒型，遺伝子組換え型）
- 非臨床試験での有効性・安全性の確認及び至適用法・用量の検討
- 腫瘍選択性
- 臨床での安全性
- 適切な動物モデル
- 体外からの排出（測定法，実測データ）

六つ目は，先ほど述べたように長期にわたる体外からの排出をどのように測定し，評価するかについての検討です．

ワークショップでは，世界各国から約 10 人の演者が講演し，それぞれの発表についての議論を取りまとめ，そのアウトプットとして見解案を作成しようと考えています．

6.3 Viral Shedding（Table 15）

2002 年に開催された第 1 回遺伝子治療ワークショップにおいて，viral shedding についての議論を行いました．

アデノウイルスベクターの体外への放出について

Table 15　Viral Shedding

- 第1回遺伝子治療ワークショップで viral shedding についても議論（2002年）
  - アデノウイルスベクターの体外への排出（Shedding）
  - ウイルスベクターの体外への排出に伴う安全性上の問題は現在までのところ確認されていない
  - アデノウイルス5型国際標準品
  - アデノウイルス5型国際標準品を使用することによって，異なる施設/研究で測定されたウイルス粒子数及び力価のデータ同士を科学的に比較することが可能となる．これにより，用量依存的な毒性のような安全性に関する情報の相関関係を明らかにすること，及び遺伝子治療用アデノウイルスベクターの製品中に含まれる増殖性アデノウイルス（RCA）の真の定量値を求めることが可能となるであろう．
- 2008年（？）ICH7 で virus/vector shedding について総合的に議論（予定）

Table 16 ICH遺伝子治療専門家会議の将来的課題

- Viral Shedding
  - 測定法の確立．安全性評価．
- ウイルスベクター標準品
  - レンチウイルス（？）
- Insertional Mutagenesis
  - 挿入部位の高感度測定法開発．挿入部位が特定出来るより安全なベクターの開発．
- 腫瘍溶解性ウイルスの品質・安全性・有効性評価

は，安全性上の問題は今までのところ確認されていません．しかし，正確な評価をするためには参照品が必要ですので，アデノウイルス5型の国際標準品を使用することによって，異なる施設，若しくは研究で測定されたウイルス粒子数及び力価のデータを科学的に比較することが可能になること等について議論しました．

更に今のところ決定されているわけではないのですが，2008年に ICH7 が開催される場合に Viral/Vector Shedding に関する議論を再度行い，それに基づいて見解案を策定することをステアリングコミッティに申請しました．

Table 17　ICH GTDG国内メンバー（於横浜会議）

MHLW	JPMA
国立医薬品食品衛生研究所	日本製薬工業協会
• 山口照英	• 鳥海亙
• 内田恵理子	• 小澤健夫
医薬品医療機器総合機構	• 井上誠
• 荒戸照世	• 竹迫一任
• 前田大輔	• 玄番岳践
	• 田中舞紀

7．GTGD の将来的課題（Table 16）

遺伝子治療専門家会議の将来的課題の一つ目は，先ほど述べた Viral Shedding について，測定法が十分確立されていませんので，今後検討していく必要があります．

二つ目は，ウイルスベクター標準品について，レンチウイルス，AAV（アデノ随伴ウイルス），又はそれ以外のウイルスについて標準品が必要かどうかを検討し，必要であればどのように作成して，どのように利用するかを検討する必要があります．

三つ目は，Insertional Mutagenesis を評価するために挿入部位についての高感度測定法の開発があげられます．現在のところ専門家会議の議論でも確定的な方法はなく，複数の方法を取り合わせることによって推論するしかないとされており，より適切な方法を開発したり，あるいは挿入部が特定できるような安全なベクターの開発が望まれています．

四つ目は，腫瘍溶解性ウイルスの品質・安全性・有効性評価に関しては，見解を取りまとめる中で明らかにしたいと考えています．

最後に ICH 横浜会議に参加した MHLW，JPMA のメンバーを Table 17 に示し，謝意を表したいと思います．

文　献

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Change in Annexin A3 Expression by Regulatory Factors of Hepatocyte Growth in Primary Cultured Rat Hepatocytes

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We have recently reported that annexin (Anx) A3 expression is necessary for hepatocyte growth in cultured rat hepatocytes seeded at half the subconfluent density on collagen. In the present study, we investigated the effects of various regulatory factors of hepatocyte growth on AnxA3 expression. AnxA3 expression was significantly reduced in hepatocytes cultured under various growth inhibitory conditions such as presence of dexamethasone, culture at subconfluent cell density, and on EHS-Matrigel and lactose-carrying styrene polymer. On the other hand, hepatocyte growth factor and epidermal growth factor, stimulators of hepatocyte growth, significantly increased AnxA3 expression in hepatocytes cultured on EHS-Matrigel. These results show close correlation between known stimulatory or inhibitory actions of various factors to hepatocyte growth and increase or decrease in AnxA3 expression, and suggest the involvement of AnxA3 in their regulation of hepatocyte growth.

Key words annexin A3; hepatocyte growth; primary cultured rat hepatocyte

Annexin (Anx) A3, also called "lipocortin 3" or "placental anticoagulant protein 3" (PAP-III),[1] is a member of the lipocortin/Anx family, which binds to phospholipids and membranes in a Ca^{2+}-dependent manner.[2,3] AnxA3 has been shown to have anticoagulant and anti-phospholipase A_2 properties *in vitro*[4] and to promote the Ca^{2+}-dependent aggregation of isolated specific granules from human neutrophils.[5] However, physiological functions have been completely unknown.[6] AnxA3 has been detected in lung, spleen, placenta, and adrenal medulla, but not in liver and isolated hepatocytes.[7—11]

We have recently reported that AnxA3 is expressed in cultured rat hepatocytes and that inhibition of AnxA3 expression by RNA interference results in a significant inhibition of hepatocyte growth.[12] This evidence indicates that AnxA3 acts as a positive regulator on hepatocyte growth in cultured hepatocytes. In relation to our report, it is noteworthy that hepatocytes placed under culture conditions acquire a growth potential characterized by enhancement of hepatocyte growth dependent on several growth factors,[13—15] whereas adult hepatocytes are normally quiescent *in vivo*.[16,17] The correlation between AnxA3 expression and growth potential of hepatocytes described above suggests that AnxA3 is one of the factors necessary for hepatocytes placed under culture to acquire growth potential.

On the other hand, hepatocyte growth is regulated in cultured hepatocytes by various other factors including cell density,[18] humoral factors such as dexamethasone (Dex),[19—21] hepatocyte growth factor (HGF) and epidermal growth factor (EGF),[14] and cellular substratum such as EHS-Matrigel[22] and lactose-carrying styrene polymer (PVLA).[23] In relation to our report, the question of whether regulation of hepatocyte growth by these factors could be mediated by concurrent change in AnxA3 expression seemed interesting; however, it remained to be elucidated whether these factors cause change in AnxA3 expression.

In the present study, we investigated the effects of various regulators of hepatocyte growth on the AnxA3 expression to examine the involvement of AnxA3 in their regulation of hepatocyte growth.

MATERIALS AND METHODS

Materials Recombinant human hepatocyte growth factor (HGF) was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). Mouse epidermal growth factor (EGF) was purchased from Wako Pure Chemical, Ltd. (Osaka, Japan). Porcine dermal collagen type 1 (collagen) was purchased from Koken Co. (Tokyo, Japan). Lactose-carrying styrene polymer (PVLA) was purchased from Seikagaku Corp. (Tokyo, Japan). Dishes (10 cm) precoated with Matrigel were purchased from BD Biosciences (Bedford, MA, U.S.A.). Rabbit anti-human AnxA3 serum was a gift from Dr. F. Russo-Marie and Dr. C. Ragueness-Nicol. Rabbit anti-rat albumin IgG and rabbit anti-rat β-actin IgG were purchased from Cappel (Aurora, Ohio, U.S.A.) and Biolegend, Inc. (San Diego, CA, U.S.A.), respectively.

Cell Isolation and Monolayer Cultures Parenchymal hepatocytes were isolated from adult male Wistar rats weighing 180—200 g, by *in situ* perfusion of the liver with collagenase.[24] All animal care and procedure protocols were approved by the institutional animal care committee. The cells were then suspended at a density of 2.5×10^5 cells/ml or 5.0×10^5 cells/ml in Williams medium E (WE) containing 5% fetal bovine serum, 1 nM insulin and 1 μg/ml aprotinin, and cultured at a density of 0.5×10^5 cells/cm^2 or 1.0×10^5 cells/cm^2 in a 10-cm dish precoated with 0.03% collagen. Alternatively, the cells were then suspended at a density of 2.5×10^5 cells/ml in WE containing 1 nM insulin and 1 μg/ml aprotinin and cultured at a density of 0.5×10^5 cells/cm^2 in a 10-

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cm dish precoated with Matrigel or a 10-cm dish precoated with 100 μg/ml PVLA. The cells were cultured in a humidified chamber at 37 °C in 5% CO_2 and 30% O_2 in air. After 2.5 h of culture, the medium was replaced with a serum- and hormone-free medium containing aprotinin (1 μg/ml), and then various humoral factors to be tested were added and the cells were further cultured for 1 d. After 1 d culture, the medium was replaced as described above, and then the humoral factors were again added and the cells were further cultured for 1 d.

Western Blot Analysis Cell lysates were prepared from the cells 2 d after the start of culture by modification of a method previously described.[25] The cells were washed twice with 5 ml of phosphate-buffered saline and then once with 5 ml of buffer A (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 10 mM EDTA). The cells were then harvested after the addition of 20 μl of buffer A. The cells were suspended, shaken for 15 min at room temperature, and sonicated four times for 15 s each time while in an ice bath after the addition of 1/5 [v/v] of 5×buffer A containing 2.5% Triton X-100 and 1/100 [v/v] of a protease inhibitor cocktail (SIGMA). After centrifugation at 100000×g, an equal amount of cytosolic protein in each experiment was subjected to SDS-PAGE on a 10% gel and electroblotted to a PVDF membrane (GVHP; Millipore). After blocking the membrane with 5% skimmed milk, a Western blot analysis was performed using rabbit anti-human AnxA3 antibody serum at a dilution of 1 : 21000, rabbit anti-rat albumin IgG at a dilution of 1 : 80000, or rabbit anti-rat β-actin IgG at a dilution of 1 : 500. Detection was performed using an ECL detection system (Amersham Bioscience). We used albumin or β-actin as a housekeeping protein based on the results of the preliminary studies. The intensity of each band was measured over a proportional range in the experiments. A computer assisted-analyzer was used to quantitatively analyze the intensity, and the intensity of each AnxA3 band was normalized to the intensity of the housekeeping protein.

Total RNA Extraction and Real-Time Quantitative PCR Total RNA was extracted from the cells 1 d after the start of culture using Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. An equal amount of RNA (approximately 1 μg) in each experiment was reverse-transcribed using a THERMOSCRIPT™ RT-PCR System (Invitrogen) and oligo$(dT)_{20}$ at a final volume of 40 μl in accordance with the manufacturer's protocol, and then diluted two-fold with ultrapure water. Subsequently, 2 μl of cDNA was used as a template for real-time PCR analysis in a Light-Cycler system (Rosche), in accordance with the manufacture's instructions. For AnxA3 and albumin, the PCR program consisted of 40 cycles of 10 s at 94 °C, 10 s at 60 °C, and 12 s at 72 °C. For 18S rRNA, the PCR program consisted of 40 cycles of 10 s at 94 °C, 10 s at 60 °C, and 20 s at 72 °C. The sequences of AnxA3-specific primers were 5′-CAA-ATTCACCGAGATCCTGT-3′ and 5′-TGCTGGAGTGCTG-TACGAAA-3′,[12] those of the albumin-specific primers were 5′-AAGGCACCCCGATTACTCCG-3′ and 5′-TGCGAAGT-CACCCATCACCG-3′,[26] and those of 18S rRNA-specific primers were 5′-CCAGAGCGAAAGCATTTGCCA-3′ and 5′-GGCATCACAGACCTGTTATTGCTC-3′. The 18S rRNA PCR product specificity was confirmed by DNA sequencing using an ABI Prism 377 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analyses. We used albumin or 18S rRNA as a housekeeping gene based on the results of the preliminary studies. And the AnxA3 levels were normalized to the levels of the housekeeping gene.

RESULTS

Effects of Dex and HGF on AnxA3 Expression by Hepatocytes Cultured on Collagen We investigated the effect of Dex and HGF on AnxA3 expression by hepatocytes cultured on collagen. Dex (100 nM) suppressed the increase of AnxA3 protein level during culture by approximately 80% (Fig. 1). On the other hand, HGF (20 ng/ml) had no effect on the AnxA3 protein level (Fig. 1). Dex (100 nM) suppressed the increase of AnxA3 mRNA level by approximately 80% (Fig. 2).

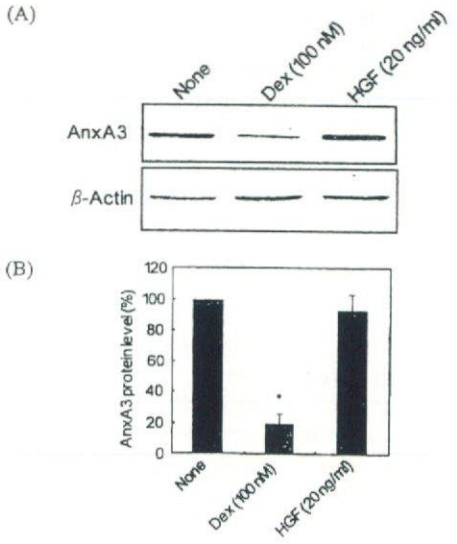

Fig. 1. Effects of Dex and HGF on AnxA3 Protein Level by Hepatocytes Cultured on Collagen

(A) The data shown are representative of the Western blot analysis results. Approximately 7.5 μg of protein was used for the detection of AnxA3 and β-actin. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen in the absence of humoral factors, shown as None. The data are expressed as the mean±S.D. of 3 experiments. * $p<0.01$, compared with the value of None.

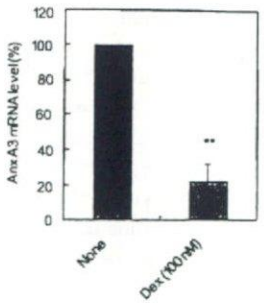

Fig. 2. Effects of Dex on AnxA3 mRNA Level by Hepatocytes Cultured on Collagen

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean±S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen in the absence of humoral factors, shown as None. ** $p<0.05$, compared with the value of None.

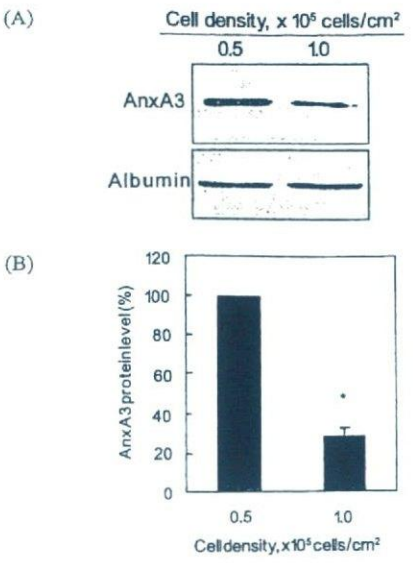

Fig. 3. AnxA3 Protein Level by Hepatocytes Cultured on Collagen at High Density

(A) The data shown are representative of the Western blot analysis results. Approximately 30.0 and 7.5 μg of protein were used for the detection of AnxA3 and albumin, respectively. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. The data are expressed as the mean±S.D. of 3 experiments. $*p<0.01$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

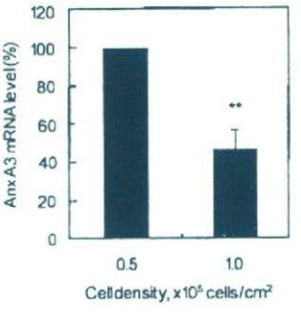

Fig. 4. AnxA3 mRNA Level by Hepatocytes Cultured on Collagen at High Density

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean±S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. $**p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

Effect of Cell Density on AnxA3 Expression by Hepatocytes Cultured on Collagen We investigated the effect of cell density on AnxA3 expression by hepatocytes cultured on collagen. AnxA3 protein and mRNA levels produced by the hepatocytes seeded at subconfluent cell density (1×10^5 cells/cm^2) were approximately 70% and 50% lower than those by the hepatocytes seeded at half of subconfluent cell density (0.5×10^5 cells/cm^2), respectively (Figs. 3, 4).

AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel and PVLA We investigated AnxA3 expression by hepatocytes cultured on EHS-Matrigel and PVLA, and compared the expression with that by hepatocytes cultured on collagen. AnxA3 protein was not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). And AnxA3 protein level produced by hepatocytes cultured on PVLA was approximately 70% lower than that by hepatocytes cultured on collagen (Fig. 5). mRNA levels produced by hepatocytes cultured

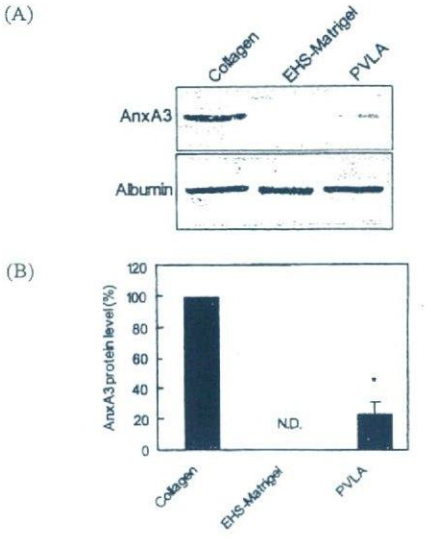

Fig. 5. AnxA3 Protein Level by Hepatocytes Cultured on EHS-Matrigel and PVLA

(A) The data shown are representative of the Western blot analysis results. Approximately 20.0 and 10.0 μg of protein were used for the detection of AnxA3 and albumin, respectively. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. The data are expressed as the mean±S.D. of 3 experiments. $*p<0.01$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. N.D.: not detected.

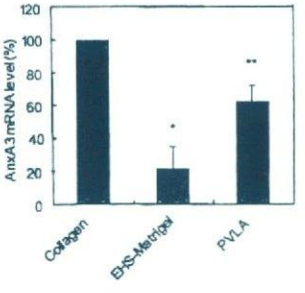

Fig. 6. AnxA3 mRNA Level by Hepatocytes Cultured on EHS-Matrigel and PVLA

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean±S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen. $*p<0.01$, $**p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on collagen.

on EHS-Matrigel and PVLA were approximately 80% and 40% lower than that by hepatocytes cultured on collagen, respectively (Fig. 6).

Effect of HGF and EGF on AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel We investigated the effect of HGF and EGF on AnxA3 expression by hepatocytes cultured on EHS-Matrigel. HGF and EGF significantly increased AnxA3 protein level, from an initially undetectable level (Fig. 7). This result suggests that the failure of increase of AnxA3 protein level by HGF, as shown in Fig. 1, is due to maximal stimulation of AnxA3 expression in hepatocytes cultured on collagen. In relation to these findings, it is seen that the stimulation of AnxA3 expression in hepatocytes cultured on collagen is not due the stimulation of HGF synthesis, because hepatocytes do not synthesize HGF.[27] HGF and EGF also increased AnxA3 mRNA levels by approximately 3.2-fold and 2.5-fold (Fig. 8), respectively.

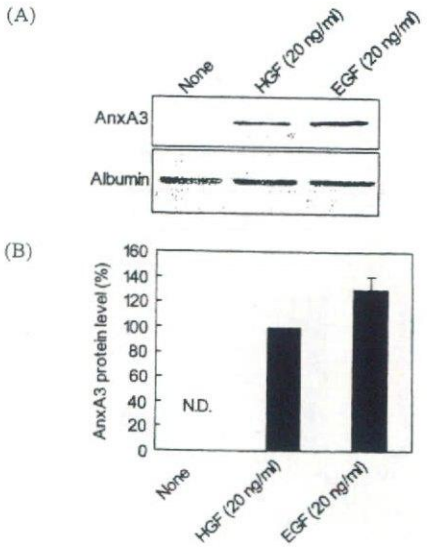

Fig. 7. Effects of HGF and EGF on AnxA3 Protein Level by Hepatocytes Cultured on EHS-Matrigel

(A) The data shown are representative of the Western blot analysis results. Approximately 10.0 μg of protein was used for the detection of AnxA3 and albumin. (B) The value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors is shown as None. The results are shown relative to the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the presence of HGF. The data are expressed as the mean±S.D. of 3 experiments.

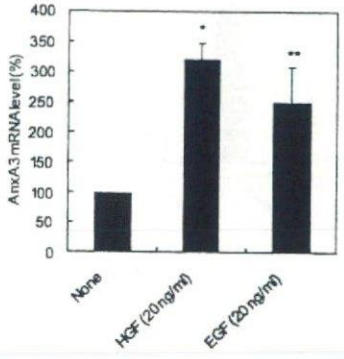

Fig. 8. Effects of HGF and EGF on AnxA3 mRNA Level by Hepatocytes Cultured on EHS-Matrigel

The AnxA3 levels were normalized to the levels of a housekeeping gene, 18S rRNA. The data are expressed as the mean±S.D. of 3 experiments. The value produced by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors is shown as None. The results are shown relative to the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors. * $p<0.01$, ** $p<0.05$, compared with the value by hepatocytes cultured at the cell density of 0.5×10^5 cells/cm^2 on EHS-Matrigel in the absence of humoral factors.

DISCUSSION

In the present study, we showed that AnxA3 expression is changed by various factors of hepatocyte growth. These factors can be classified as stimulators and inhibitors of hepatocyte growth. EGF and HGF are typical stimulators of hepatocyte growth in cultured hepatocytes.[14] Other factors belong to the inhibitors group, as follows: Dex suppresses hepatocyte growth in cultured hepatocytes.[19—21] Hepatocytes cultured at high density show lower levels of hepatocyte growth compared with those cultured at low density.[18] Hepatocytes cultured on EHS-Matrigel[22] and PVLA[23] show extremely low levels of hepatocyte growth compared with those cultured on collagen and plastic dishes. From the present findings it is evident that AnxA3 expression is increased and decreased in concurrent with enhancement and suppression of hepatocyte growth by growth stimulatory and inhibitory factors, respectively. The close correlation between known actions of various stimulators and inhibitors on hepatocyte growth and change in AnxA3 expression is consistent with our recent finding that AnxA3 acts as a positive regulator of hepatocyte growth in cultured hepatocytes.[12] In addition, we discovered that enhanced expression of AnxA3 was observed in the proliferative hepatocytes after carbon tetrachloride-induced rat liver damage and 70% partial hepatectomy (unpublished observation). This may lead to speculation that regulation of AnxA3 expression by these factors may be involved in their regulation of hepatocyte growth.

The most marked observation in the present study seems to be that AnxA3 protein is not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). Interestingly, hepatocytes cultured on EHS-Matrigel show small round-shaped morphology compared with those cultured on the collagen, resembling those *in vivo*,[22] whereas hepatocytes cultured on the collagen were uniformly spread flat. This evidence suggests that AnxA3 expression is dramatically reduced in cultured hepatocytes that show round-shaped morphology. This possibility may be supported by the finding that inverse correlation between DNA synthesis and roundness of hepatocytes is observed in cultured hepatocytes showing various morphologies from round shape to flat shape by coating dishes with different concentrations of PVLA.[23] Although hepatocytes cultured on PVLA also show small round-shaped morphology,[23] reduction of AnxA3 protein under this condition is not so marked compared with that using EHS-Matrigel (Fig. 5). Careful microscopic examination showed that morphology of hepatocytes cultured on EHS-Matrigel is smaller and rounder compared with that of hepatocytes cultured on PVLA (data not shown).

There are many reports showing that various factors regulate expression of other Anxs. For example, Dex and other glucocorticoids stimulate AnxA1 expression *in vitro*[28—32] and *in vivo*,[33—36] in contrast to the present finding. This inconsistency suggests that the mode of regulation by glucocorticoids differs among species of Anxs, tissues, and cells. HGF and EGF stimulate expression of AnxA1, AnxA2, AnxA5, and AnxA6 in primary cultured rat hepatocytes.[37] Interleukin-6[38] and 12-*O*-tetradecanoylphorbor β-acetate[39] stimulate AnxA1 expression in A549 cells and cultured astrocytes, respectively.

In conclusion, the present study shows a close correlation between the known actions of various factors to hepatocyte growth and change in AnxA3 expression, and suggests that the changes in AnxA3 expression associated with these factors could be involved in their regulation of hepatocyte growth.

Acknowledgement This work was supported in part by the Gant-in-Aid for Cancer Research (15-2) from the Ministry of Health, Labor and Welfare.

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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).

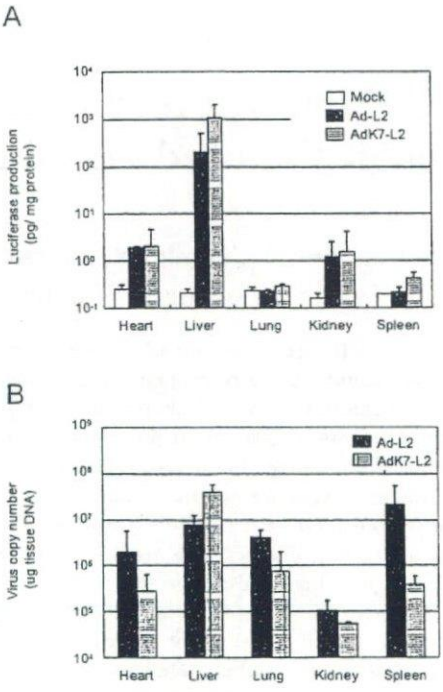

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production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

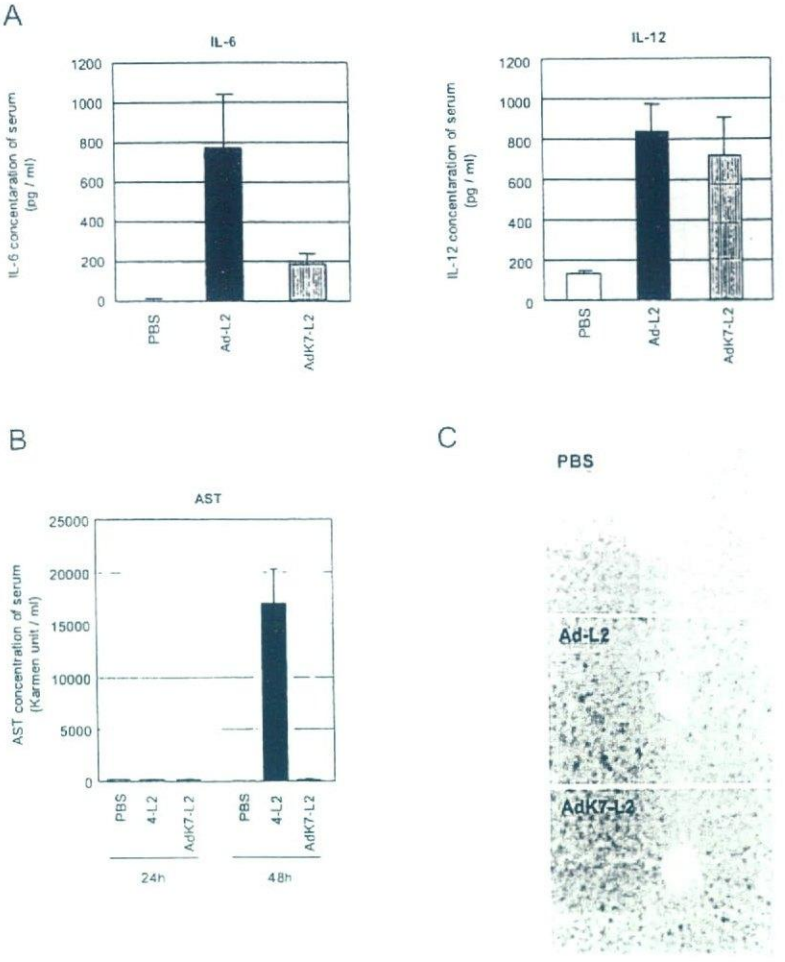

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*).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

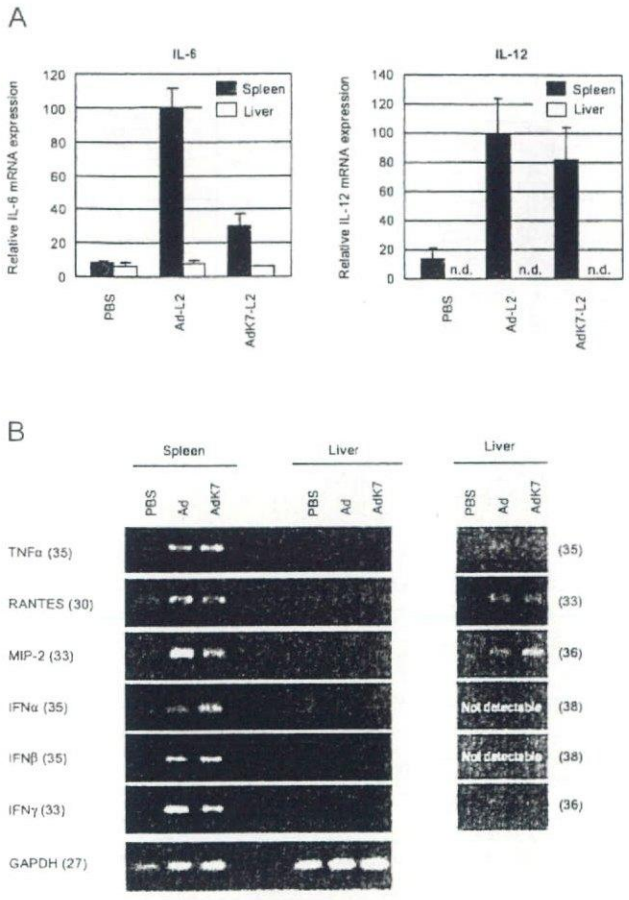

FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction,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

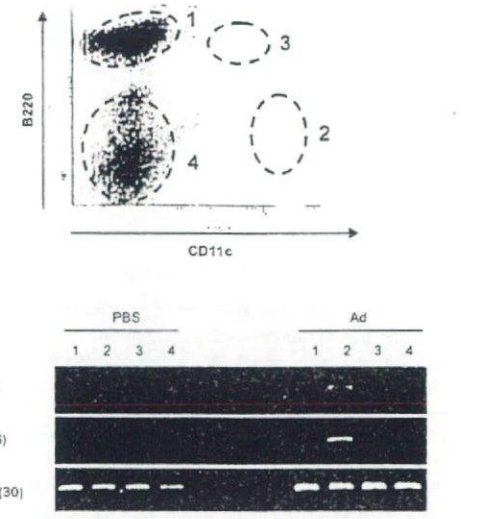

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by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

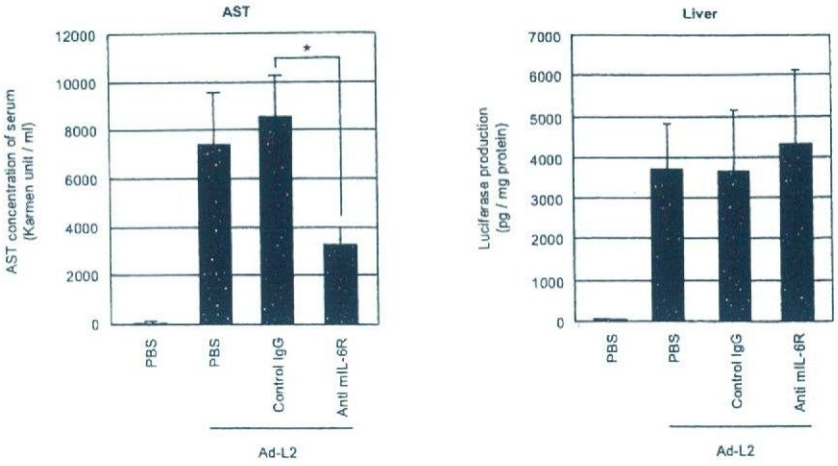

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; 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.

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Disclosures

The authors have no financial conflict of interest.

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SHORT COMMUNICATION

Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon

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Adenovirus (Ad) vectors are widely used in gene therapy and in vitro*/*in vivo *gene transfer because of their high transduction efficiency. However, Ad vector application in the gene therapy field is limited by poor transduction into cells not expressing the primary receptor, coxsackievirus and adenovirus receptor. To overcome this problem, several types of capsid-modified Ad vectors have been developed. The HI loop or C-terminus of the fiber knob, the C-terminus of the protein IX (pIX) and the hypervariable region 5 of the hexon are promising candidate locations for displaying foreign peptide sequences. In the present study, we constructed Ad vectors in which each of the above region was modified by a simple* in vitro *ligation-based method, and examined the characterization of each Ad vector containing the FLAG tag (DYKDDDDK) or RGD (CDCRGDCFC) peptide. Enzyme-linked immunosorbent assay examining the surface expression of foreign peptides on the virus suggested that foreign peptides are exposed on virion surfaces in all types vectors and that the hexon was the most efficiently reacted, reflecting the copy number of the modification. However, in the case of the transduction efficiency of Ad vectors containing the RGD peptides, the modification of pIX and the hexon showed no effect. The modification of the HI loop of the fiber knob was the most efficient, followed by the modification of the C-terminus region of the fiber knob. These comparative analyses, together with a simple construction method for each modified Ad vector, could provide basic information for the generation of capsid-modified Ad vectors.*
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Introduction

Adenovirus (Ad) vectors based on Ad type 5 are widely used for gene transfer studies and clinical gene therapy trials, since they can achieve high transduction efficiency and transduce into both dividing and non-dividing cells.[1,2] However, one of the hurdles confronting Ad-mediated gene transfer is that Ad infection is dependent on the expression levels of the coxsackievirus and adenovirus receptor (CAR) in the target cells. Ad vectors cannot transfer genes of interest into cells lacking CAR expression (i.e. many advanced tumor cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, etc).[3,4]

Genetic modification of the Ad capsid, such as its fiber, protein IX (pIX), or hexon, is an attractive strategy for altering the Ad tropism. Among these options, modification of the fiber proteins has been the most widely studied. Fiber proteins consist of three distinct domains: the tail, shaft and knob. Each domain has distinct functions in host cell infection. The trimeric subunits of the C-terminal knob domain are responsible for binding to the host's primary cellular receptor, CAR.[5,6] Incorporation of the RGD (Arg-Gly-Asp) peptide or a stretch of lysine residues (K7 [KKKKKKK] peptide), which target αv integrins or heparan sulfate proteoglycans on the cellular surface, respectively, into the HI loop or the C-terminal region of the fiber knob allows Ad tropism to be expanded (or changed) by binding the modified fiber protein with the cellular receptor.[7–10]

The C-terminal region of pIX is another candidate location for capsid modification.[11] pIX is a minor structural protein contained in the Ad virion which enhances the structural integrity of the particles by stabilizing hexon–hexon interaction.[12,13] It also plays roles in transcriptional activity and nuclear reorganization.[14] The attractive characteristics of ligand insertion into the pIX region is that the C-terminus of pIX tolerates the insertion of large ligands.[15–17] As Ad pIX resides at a deep and hidden position below the tops of the hexon capsomer, Ad vectors containing the ligand-pIX fusion protein, which incorporates an α-helical spacer to sufficiently lift the ligands and expose them at the surface of the capsid, were developed.[18] Enhanced transduction was reported by Ad vectors containing the RGD peptide in the C-terminus of pIX with an α-helical spacer.[18]

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Hexons are the most abundant capsid proteins and comprise each geometrical face of the capsid. As hexons are mostly targeted by neutralizing antibodies,[19] hexon modification has been reported to escape from neutralizing antibodies as well as to modify the tropism.[20] The hypervariable region (HVR) 5 of hexon loop L1 is a candidate location for incorporating foreign peptides without affecting the normal function of Ad type 5 as a gene transfer vector (i.e. viral growth, virus formation, virion stability, CAR-mediated infectivity).[21,22] Vigne *et al.*[21] have reported that Ad vectors containing the RGD peptides (DCRGDCF) at HVR5 of the hexon can infect cells via cellular αv integrin, independently of the CAR. However, Wu *et al.*[22] have reported that the His tag sequence at HVR5 had no effect on the transduction efficiency of Ad vectors when the vectors were applied to cells expressing anti-His tag single-chain antibody (scFv).

One attractive point of pIX or hexon modification is that 240 or 720 molecules of foreign peptides per virion are displayed at pIX or the hexon, respectively, while only 36 molecules are displayed at the fiber (note that the fiber and hexon are composed of trimeric subunits). Therefore, pIX- or hexon-modified Ad vectors containing heterologous peptides might be more effective than fiber-modified Ad vectors.

In the present study, we first developed a simple method for generating pIX- or hexon-modified Ad vectors by using *in vitro* ligation-based plasmid construction. The functionalities of Ad vectors containing the FLAG tag or RGD peptide in the HI loop or C-terminus of the fiber knob, C-terminus of pIX, or the HVR 5 region of the hexon were systemically compared. These comparative analyses could provide basic information for generation of capsid-modified Ad vectors.

First, we constructed newer vector plasmids pAdHM56 and -62, which we used for the generation of pIX- or hexon-modified Ad vectors, respectively. These contain a unique *Xba*I site in the coding regions of the C-terminal of pIX or HVR5 of the hexon, respectively (Figure 1), where only a minimal *Xba*I recognition sequence (TCTAGA) was introduced. pAdHM56 and -62 also contain unique I-*Ceu*I/*Swa*I/PI-*Sce*I sites in the E1 deletion region; hence, a transgene expression cassette can be cloned into the E1 deletion region by simple *in vitro* ligation, as previously described.[23,24] The shuttle plasmid pHM15-75A was constructed by introducing oligonucleotide 1/2, −3/4, −5/6, −7/8 (Table 1) into a derivative plasmid of pHM15.[25] pHM15-75A contains *Xba*I, *Avr*II, *Nhe*I and *Spe*I sites at both ends of a multicloning site and the coding sequence of a 75 Å α-helical spacer, as described by Vellinga *et al.*,[18] in the same multicloning site (Figure 1a). Detailed information about the constructions of the vector and shuttle plasmids is available from the authors upon request.

We previously developed a simple method for generating fiber-modified Ad vectors.[9,10] In that method, unique restriction enzyme sites (*Csp45*I and/or *Cla*I site) were introduced into the HI loop or C-terminal coding region of the fiber knob of the Ad vector plasmid, and the foreign DNA coding peptide of interest could easily be cloned into their regions by simple *in vitro* ligation.

In the present study, we expanded upon this system, so that heterologous peptide sequences could be inserted at the C-terminus region of pIX and the HVR5 region of the hexon. Figure 1 shows a representative example for generating Ad-RGD(pIX/75)-L2 and Ad-RGD(hexon)-L2, luciferase-expressing Ad vectors containing the RGD peptides in the C-terminus of the pIX via a 75 Å α-helical spacer and in the HVR5 region of the hexon, respectively. By using pHM15-75A, the peptide of interest coding sequence together with the 75 Å α-helical spacer coding sequence could easily be introduced into the C-terminal coding region of pIX of pAdHM56. As *Xba*I, *Avr*II, *Nhe*I and *Spe*I produce compatible cohesive ends, *Avr*II, *Nhe*I and *Spe*I sites as well as the *Xba*I site can be used for cloning into the *Xba*I site. Oligonucleotides corresponding to the peptide of interest can also be directly introduced into the *Xba*I site of pAdHM56. For the generation of pAdHM56-RGD75-L2, *Xba*I-digested pAdHM56-L2, in which a luciferase expression cassette was introduced into the E1 deletion region of pAdHM56 using I-*Ceu*I and PI-*Sce*I sites,[23,24] was ligated with *Avr*II-digested pHM15-75A-RGD, which was constructed by the introduction of oligonucleotides 9/10 corresponding to a GS linker plus the RGD-4C (CDCRGDCFC) peptide into the *Csp45*I/*Asc*I sites of pHM15-75A (Figure 1a). pAdHM62-RGD-L2, a vector plasmid for the hexon-modified Ad vector containing the RGD peptide, was constructed by the ligation of *Xba*I-digested pAdHM62-L2 with oligonucleotides 11/12, which contain a binding site with an *Xba*I-digested fragment and correspond to the RGD-4C peptide (Figure 1b). Oligonucleotides were designed so that the positive recombinant plasmid lacked an *Xba*I site for convenience of selection. Sequencing of all inserted oligonucleotides in each plasmid verified that the clones contained the appropriate sequences.

pAdHM56-FLAG-L2, pAdHM56-FLAG75-L2, pAdHM56-His-L2, pAdHM56-His75-L2, pAdHM56-RGD-L2 and pAdHM62-FLAG-L2 were similarly constructed as shown in Figure 1 by using *Xba*I-digested pAdHM56-L2 or pAdHM62-L2 with oligonucleotides 11–20. Ad vectors were generated by the transfection of *Pac*I-digested linearized vector plasmids described above into 293 cells and were prepared as described previously.[23,24] The conventional Ad vector (Ad-L2) and fiber-modified Ad vectors (Ad-RGD(HI)-L2 and Ad-RGD(C)-L2), had been previously constructed.[9,10] Determination of virus particle titers (VP) and infectious titer was accomplished spectrophotometrically by the method of Maizel *et al.*[26] and by using an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA, USA), respectively. The infectious titer-to-particle ratio was 1:10 for Ad-L2, 1:17 for Ad-RGD(HI)-L2, 1:28 for Ad-RGD(C)-L2, 1:9 for Ad-RGD(pIX)-L2, 1:23 for Ad-RGD(pIX/75)-L2, 1:44 for Ad-RGD(hexon)-L2. All capsid-modified Ad vectors except Ad-RGD(hexon)-L2 were readily propagated, with similar particle titers to those of the conventional Ad vector, Ad-L2. The growth rate of Ad-RGD(hexon)-L2 was similar to that of Ad-L2, but the yield was approximately 10 times lower. Ad vectors and vector plasmids used in the present study are summarized in Table 2. All capsid-modified Ad vectors contain CAR and integrin binding motifs in the fiber and the penton base.

We then examined whether foreign peptides are displayed in the HI loop of the fiber knob, the C-terminus of the fiber knob, the C-terminus of pIX or the HVR5 region of the hexon (Figure 2). To do this, we generated capsid-modified Ad vectors containing the

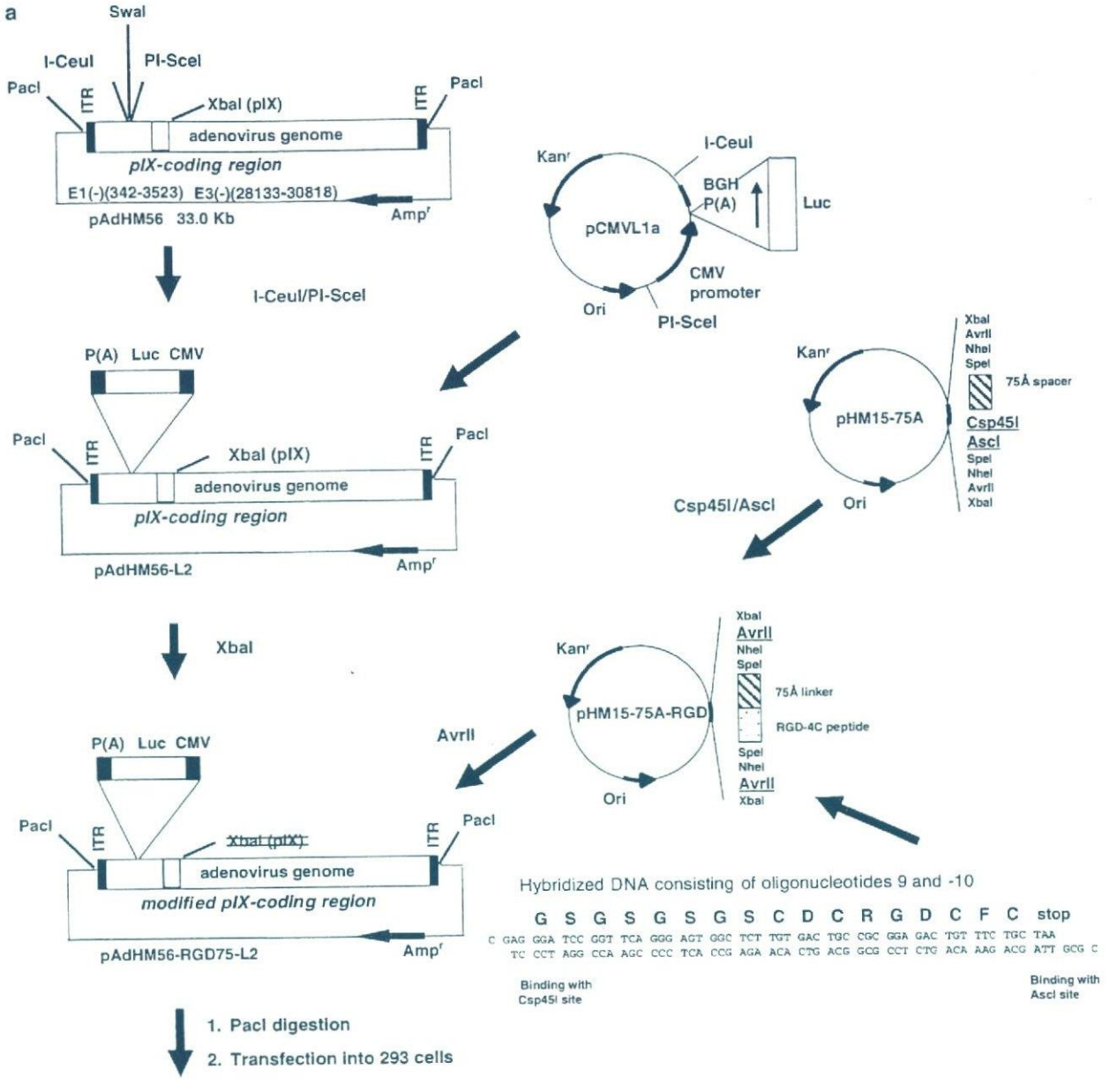

Figure 1 The construction strategy for pIX- or hexon-modified Ad vectors containing foreign peptides. (a) Construction of pIX-modified Ad vector. pAdHM56 was digested by I-*Ceu*I/PI-*Sce*I and ligated with I-*Ceu*I/PI-*Sce*I-digested pCMVL1a, which contains a CMV promoter-driven luciferase expression cassette, resulting in pAdHM56-L2. The shuttle plasmid pHM15-75A-RGD, which cloned oligonucleotides corresponding to the GS linker plus the RGD-4C peptide into pHM15-75A, was digested with *Avr*II and ligated with *Xba*I-digested pAdHM56-L2, resulting in pAdHM56-RGD75-L2. When *Avr*II, *Nhe*I or *Spe*I sites of the shuttle plasmid are used for cloning into the *Xba*I site of the vector plasmid, the positive recombinant plasmid lacks an *Xba*I site. Therefore, generation of the self-ligated plasmid is reduced by the digestion of the ligation sample with *Xba*I. A luciferase-expressing Ad vector containing the RGD peptide in the C-terminal of pIX with a 75 Å α-helical spacer, Ad-RGD(pIX/75)-L2, was produced by transfection of the *Pac*I-digested pAdHM56-RGD75-L2 into 293 cells. (b) Construction of hexon-modified Ad vector. pAdHM62-L2 was constructed by the ligation of I-*Ceu*I/PI-*Sce*I-digested pAdHM62 and I-*Ceu*I/PI-*Sce*I-digested pCMVL1a. Then, pAdHM62-L2 was digested with *Xba*I and ligated with an oligonucleotide corresponding to the linker (GS) and the RGD-4C peptide that contains a binding site with an *Xba*I-digested fragment, resulting in pAdHM62-RGD-L2. The oligonucleotide was designed so that the positive recombinant plasmid lacks an *Xba*I site. Generation of the self-ligated plasmid was reduced by the digestion of the ligation sample by *Xba*I. A luciferase-expressing Ad vector containing the RGD peptide in the HVR5 region of the hexon, Ad-RGD(hexon)-L2, was produced by transfection of the *Pac*I-digested pAdHM62-RGD-L2 into 293 cells.

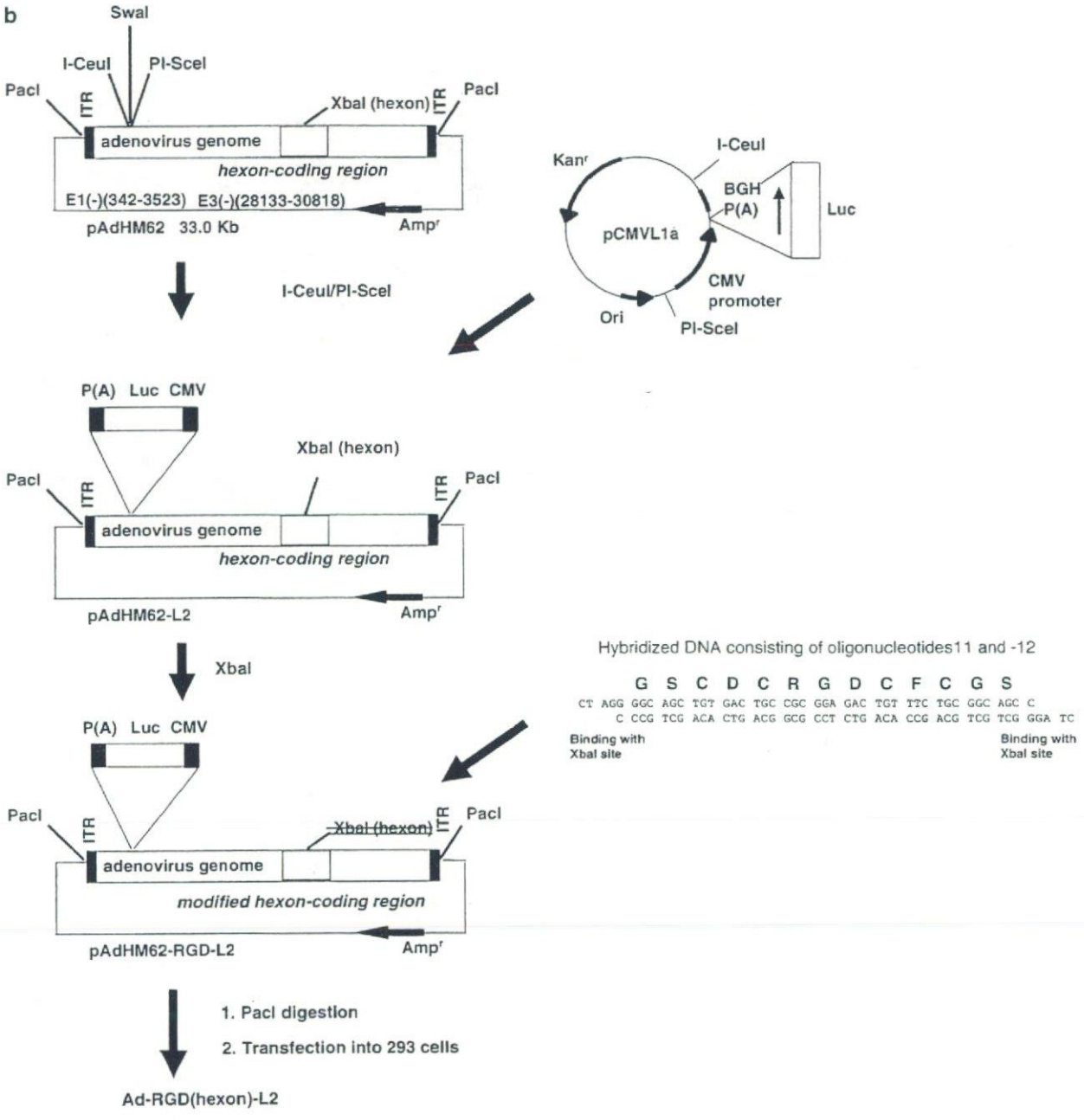

Figure 1 *Continued*

FLAG tag peptide in each region. Expression of the FLAG tag peptide in Ad-FLAG(HI)-L2, Ad-FLAG(C)-L2, Ad-FLAG(pIX)-L2 and Ad-FLAG(hexon)-L2 was examined by Western blotting. The total protein (1 μg) of each vector in 1 × sample buffer containing 4% β-mercaptoethanol was loaded on the SDS-PAGE gel after boiling 5 min, followed by electrotransfer to a PVDF (polyvinylidene difluoride) membrane. After blocking in Block Ace (Dainippon Pharmaceuticals, Osaka, Japan), the filters were incubated with ANTI-FLAG M2 monoclonal antibody (Sigma, Saint Louis, USA) (1:3000), followed by incubation in the presence of goat anti-mouse IgG HRP (Horseradish peroxidase)-linked antibody (Cell Signaling Technology Inc., MA, USA). The filters were developed by Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan). The signals were read using a LAS-3000 machine (FUJIFILM, Tokyo, Japan). The FLAG tag peptide in the HI loop and the C-terminus of the fiber knob were about 60 kDa, similar size to a fiber protein. The molecular weight of pIX was 14.4 kDa and the FLAG tag peptide of Ad-FLAG(pIX)-L2 was about 14.4 kDa. The FLAG tag peptide detected in Ad-FLAG(hexon)-L2 was about 110 kDa, which is similar to the molecular weight of the hexon. Although the copy number of pIX is higher