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.

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).

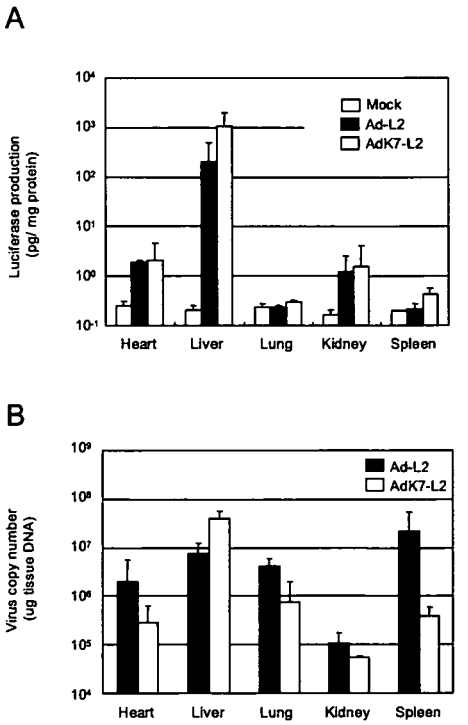

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production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

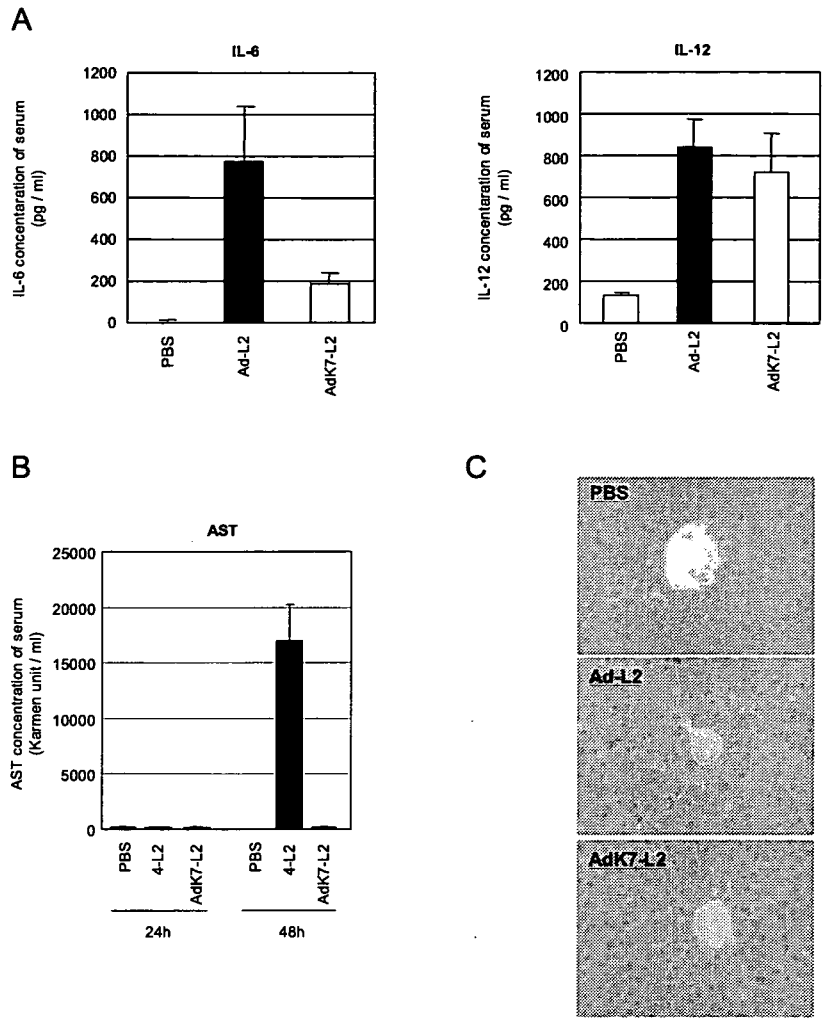

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*).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

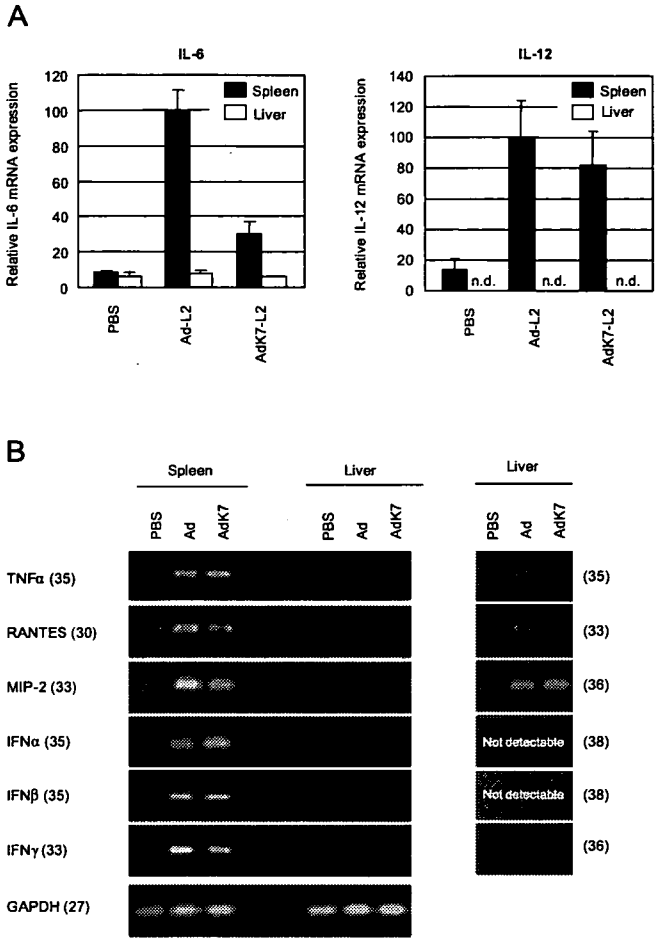

FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction,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

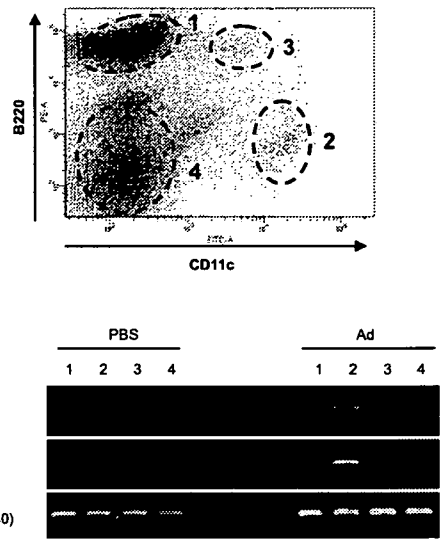

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by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

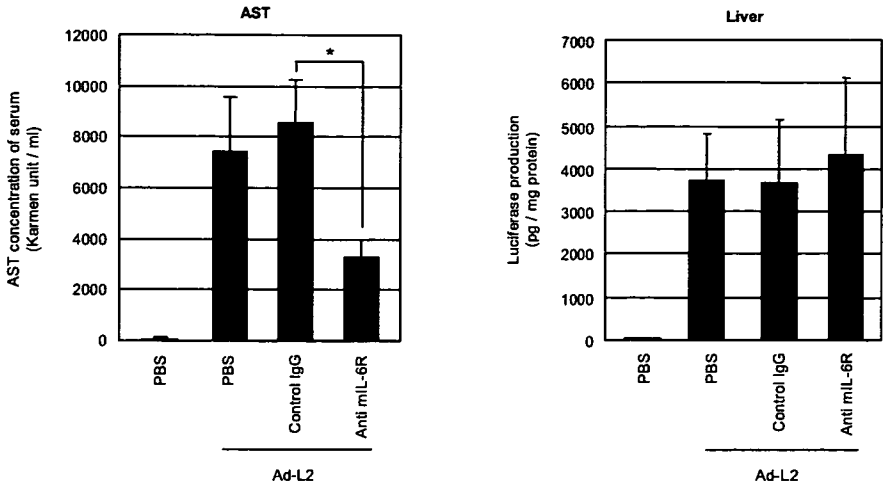

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone: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

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Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex

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Abstract

Vectors for gene expression are the essential tools for both gene therapy and basic research. There are two groups of gene therapy vectors, viral and non-viral vectors. At present, toxicity triggered by vectors is one of the major concerns for clinical trials. In general, non-viral vectors, such as plasmid DNA–cationic liposome complex (lipoplex), are thought to be safer than viral vectors, such as adenovirus (Ad) vector, although lipoplex is less efficient in term of gene expression than the Ad vector. However, there has been no study directly comparing the gene expression efficiency and safety of viral and non-viral vectors. Here, we present evidence that the Ad vector shows much more efficient gene expression and is safer than lipoplex, at least with respect to the innate immune response. After being systemically administered to mice, the Ad vector showed a transduction efficiency that was 2 to 5 log orders higher than that of lipoplex, depending on the organ. On the other hand, surprisingly, the administration of lipoplex produced a greater amount of inflammatory cytokines such as interleukin-6, interleukin-12, and tumor necrosis factor-α than did the administration of the Ad vector, whereas a comparable level of hepatotoxicity was induced by these vectors. The production of inflammatory cytokines induced by the injection of lipoplex was reduced when the CpG motifs were removed completely from plasmid DNA. Thus, care should be taken to ensure the innate immune response induced by gene therapy vectors, especially lipoplex.

Keywords: Adenovirus vector; Lipoplex; Gene therapy; In vivo gene expression; Innate immunity

1. Introduction

Vectors for gene expression are requisite tools not only for gene therapy but also for basic research, such as the functional analysis of novel genes. The success of gene therapy is largely dependent on gene delivery vectors, which can be categorized into two groups, viral and non-viral vectors [1]. The viral vectors, such as the adenovirus (Ad) vector, have great advantages such as high-level gene expression in a broad range of tissue, but such vectors are thought to lack safety because they are based on a pathogenic virus [2,3]. Another problem is that some kinds of viral vectors have an oncogenic function. Retrovirus and lentivirus vectors, which can insert foreign genes into the host DNA in a random manner, sometimes leads to canceration [4]. On the other hand, non-viral vectors based on plasmid DNA are thought to be safe even though their transduction efficiency is low [2].

Viruses are highly developed agents specialized in infection and the transfer of genetic material to the cells of other organisms, because infecting the host cells is an essential stage in their life cycle. On the other hand, the immune systems of mammals have evolved to counterattack the efforts of viral pathogens [5]. One of the viral vectors, the Ad vector, has several advantages, including the ability to package relatively large-sized foreign DNA, the ease with which it can be produced, and broad cell tropism [5,6]. However, many studies have shown that systemic administration of Ad vectors immediately triggers the innate immune response to elicit an acute inflammation, such as occurs with the secretion of

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inflammatory cytokines and tissue injury [5,7,8]. Because of these problems with viral vectors, non-viral vectors have gained increasing attention recently [1]. Among the non-viral vectors, the lipoplex (complexes of cationic liposome/ plasmid DNA) is the most studied and represents the most promising approach for human clinical trials [2]. However, the utility of non-viral vectors is often limited because of their low level and narrow range of exogenous gene expression [9,10]. As in the case of viral vectors, the production of inflammatory cytokines and tissue damage have been reported to be induced by the systemic injection of lipoplex even though it contains no viral components [11–15]. It has been shown that the immunostimulatory CpG motifs present in plasmid DNA are responsible for a significant portion of this acute response [16]. Plasmid DNA and bacterial DNA contain a much higher frequency of unmethylated CpG motifs than does mammalian DNA [11,15–17]. Toll-like receptor (TLR) 9 has been identified as the receptor involved in the recognition of immunostimulatory CpG motifs [18]. TLR9 is a member of the family of TLRs, which play a critical role in innate immunity. Ten family members have been identified so far, and they appear to activate NF-κB, leading to the production of inflammatory cytokines [15,18,19].

Although both viral and non-viral vectors have pathogenic profiles, no study has directly compared the transduction efficiency, especially in vivo, and safety of these vectors. Thus, we performed a comparative study of the transduction efficiency and the level of production of inflammatory cytokines after systemic injection of the Ad vector or lipoplex into mice.

2. Materials and methods

2.1. Plasmids and adenovirus vectors

The plasmid vector, pCMVL1, and the Ad vector, Ad-L2, which were constructed to express luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal, were described previously [20–22]. pCpG-mcs was purchased from Invivogen (San Diego, CA).

pCMVL1 and pCpG-mcs were amplified in DH5α and GT115, respectively, and isolated by using EndoFree Plasmid Mega Kit (QIAGEN, Valencia, CA). The concentration of lipopolysaccharide (LPS) in DNA solution was measured using the Limulus HS-F Single Test (Wako, Osaka, Japan). The amount of LPS in the DNA solution was <0.1 Endotoxin unit/ μg DNA, which is the amount endorsed by QIAGEN.

Ad-L2 was amplified in 293 cells; and purified by $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol; and stored in aliquots at −70 °C. Virus particle titer was measured spectrophotometrically as described previously [23].

2.2. Preparation of DOTAP/Chol liposome and lipoplex

DOTAP/Chol liposome and lipoplex were prepared by a modification of the method used by Li et al. [12]. Briefly, an appropriate amount of DOTAP (AVANTI Polar Lipids, Alabastar, Al) was mixed with cholesterol in chloroform at the molar ratio of 1:1. The organic solvent was evaporated to make the dried lipid film. The dried films were then hydrated in 5% dextrose solution under a 37 °C water bath to make liposome solution. The liposome solution was sonicated for 1–2 min before the lipoplex was prepared. To prepare the lipoplex, plasmid DNA was diluted with 5% dextrose, and then liposome solution was added to achieve a 9.8:1.0 weight ratio of DOTAP: DNA. The theoretical charge ratio (+/−) of the complex was 4.6. The mixture was incubated at room temperature for 10 min before injection.

2.3. In vivo gene transfer and luciferase assay

Female C57BL/6 mice (5–6 weeks) were purchased from Nippon SLC (Hamamatsu, Japan). A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} vector particle (VP)/mouse) or lipoplex (5 or 25 μg of plasmid DNA/mouse) was injected intravenously via tail vain of each mouse. An appropriate length of time after the injection, the mice were given anesthesic by diethylether and their hearts, lungs, kidneys, livers and spleens were collected. The organs were then homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris–HCl, pH 7.8). After being frozen and thawed, the homogenates were centrifuged at 15,000 ×*g* at 4 °C for 10 min, and the supernatants were collected. Luciferase activity in the supernatants was determined by using a luciferase assay system (PicaGene 5500; Toyo Inki, Tokyo, Japan). The protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA).

2.4. Cytokine measurement

An appropriate length of time after the injection, the mice were given anesthesic by diethylether and peripheral blood was collected via the inferior vena cava and fundus oculi. The peripheral blood was placed on ice for 2–3 h and then centrifuged at 15,000 rpm at 4 °C for 10 min to collect the blood serum. The cytokine concentration in serum was measured using an ELISA kit (IL-6 and IL-12p40; BD Biosciences, San Diego,CA, TNF-α; R & D Research Systems, Minneapolis, MN) according to the manufacturer's instructions. Alanine aminotransferase (ALT) activities of the blood serum were measured using the Transaminase CII-Test (Wako, Osaka, Japan).

2.5. Preparation of paraffin sections of liver

An appropriate length of time after the injection, the mice were given anesthesic by diethylether and their livers were collected. Each liver was washed, fixed in 10% formalin for 24 h at room temperature, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with hematoxylin and eosin. This process was commissioned to Applied Medical Research Laboratory (Osaka, Japan).

3. Results

3.1. Comparison of the transduction efficiency between Ad vector and lipoplex

To compare the transduction activity and safety of gene therapy vectors in vivo, we chose the Ad vector and DNA–liposome complex (lipoplex) as the viral vector and non-viral vector, respectively. We chose DOTAP/Chol as the liposome, because this has been used in many gene transduction studies in vivo, and its usability has been proven [2,10,24–26]. The mean diameter of the lipoplex, which was measured by Zetasizer (Malvern Instruments Ltd, United Kingdom) was about 350 nm (data not shown). Gel retardation assay showed that plasmid DNAs were indeed complexed with liposome (data not shown). To compare the in vivo transduction efficiency and the distribution of the gene expression from the Ad vector and lipoplex, we first analyzed the luciferase production in the organ after intravenous injection of the Ad vector (1×10^{10} or 5×10^{10} VP/mouse) or lipoplex (5 or 25 μg of plasmid DNA/mouse). The dose of the vectors injected in the present study was used in previous reports and was determined to be a limited dose that would not cause sudden death or raising of the hair [2,12]. High levels of luciferase production were obtained as a result of the injection of the Ad vector in all organs examined. On the other hand, lipoplex-mediated luciferase production was detected only in the heart and lung (Fig. 1). Luciferase production in lipoplex-injected mice was 10^{-5} to 10^{-2} lower than in Ad vector-injected mice in all organs. The luciferase production obtained from mice that were injected with lipoplex was approximately the same or slightly lower than has been shown in previous reports [2,25,27]. This difference might result from the differences in the mouse strains and liposome compositions used in the experiments. We also determined the duration of luciferase production after the intravenous injection

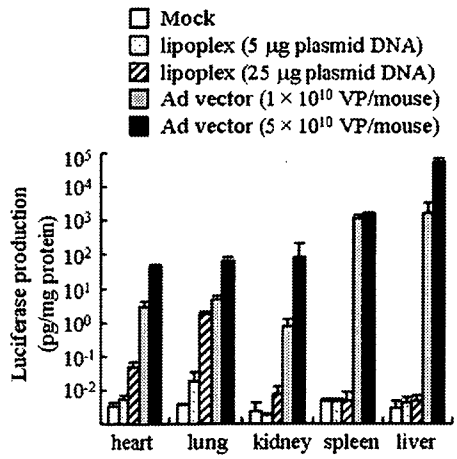

Fig. 1. Luciferase production in various organs after intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; dotted and slashed bar, respectively) was injected intravenously into each mouse. Organs were collected from the mice 6 h following the injection, and luciferase activity and protein concentration were assayed. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

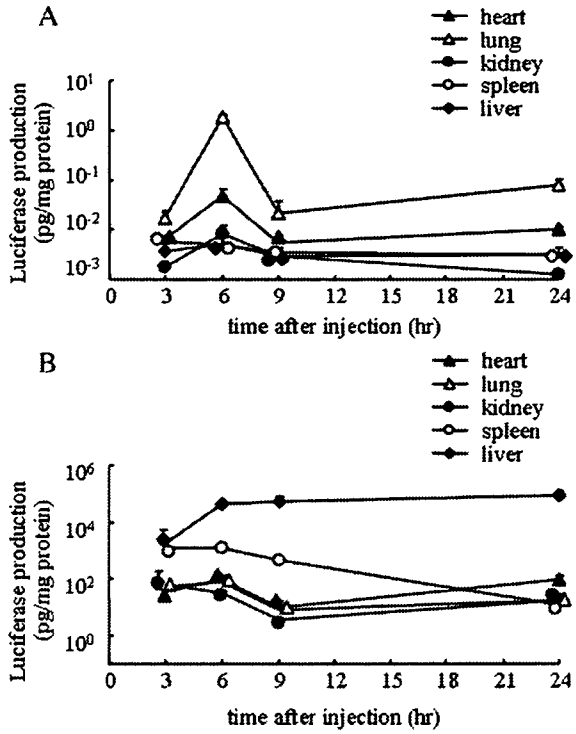

Fig. 2. Duration of the luciferase production. A final volume of 200 μl of lipoplex (25 μg of plasmid DNA; A) or Ad vectors (5×10^{10} VP/mouse; B) was injected intravenously into each mouse. After an appropriate length of time had passed following the injection, the heart (filled triangle), lung (opened triangle), spleen (opened circle), kidney (filled circle), and liver (filled diamond) were collected, and luciferase activity and protein concentration were measured. Data are expressed as means ± SD of 3–4 mice per group.

of vectors (Fig. 2A and B). Luciferase production in all organs collected from mice that were injected with the Ad vector or lipoplex decreased in a time-dependent manner. It was striking that the livers collected from mice that were injected with high doses of the Ad vector maintained a high gene expression for 24 h (Fig. 2B). On the other hand, the gene expression of lipoplex decreased dramatically and reached levels similar to that shown in mock-treated mice in most organs after 9 h (Fig. 2A). Thus, we can conclude that the Ad vector can express a high level of foreign genes in a broad range of tissues.

3.2. Vector-triggered cytokine production

There are many indices of the side effects caused by the intravenous injection of vectors, such as canceration, tissue damage, innate and adaptive immune response, etc. In the present study, innate immune response was examined as an indicator of the side effects induced by the administration of vectors, since the induction of innate immune response by the Ad vector is the primary limiting factor in the use of the Ad vector [5]. To investigate the level of immune response induced by these vectors, we measured the serum concentration of various inflammatory cytokines, including IL-6, IL-12 and TNF-α. At 6 h following the intravenous injection, the IL-6 concentration in serum from lipoplex-injected mice was 2- to 4-fold higher than that in serum from the Ad vector-injected mice (Fig. 3a). A similar profile was obtained for the production of

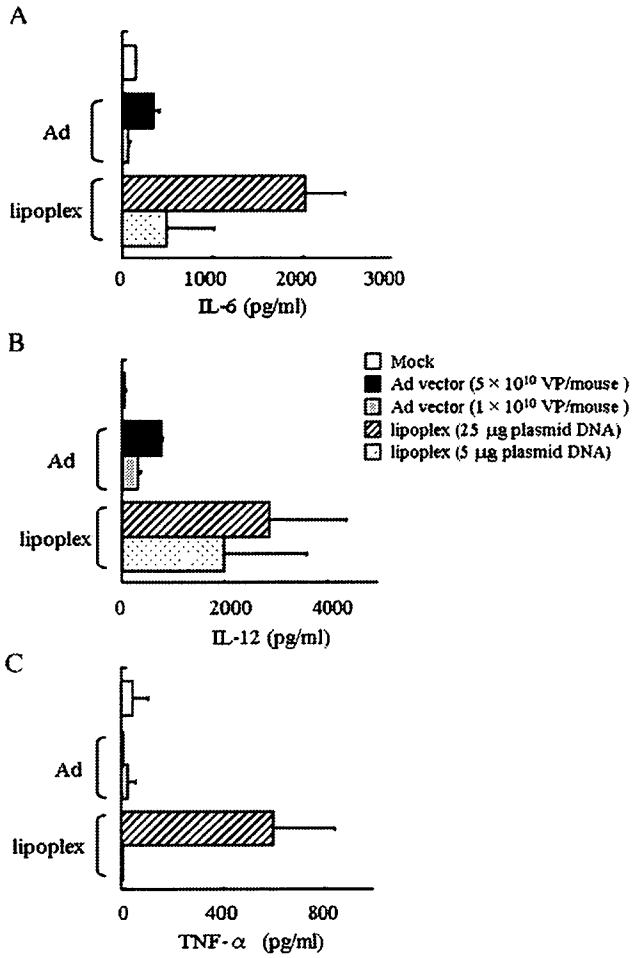

Fig. 3. Induction of various inflammatory cytokines by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected intravenously into each mouse. Six hours after the injection, peripheral blood was collected, and the concentrations of IL-6, IL-12, and TNF-α were measured by ELISA. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

IL-12 induced by these vectors (Fig. 3b). The level of cytokine production induced by these vectors was dose-dependent and returned to the basal level after 24 h post-injection (Fig. 6 shows the data for lipoplex; data not shown for the Ad vector). When a higher dose of vectors was injected, the serum concentration of TNF-α from lipoplex-injected mice was 2-orders higher than that from Ad vector-injected mice and control mice (Mock) (Fig. 3c). The serum concentration of TNF-α from the Ad vector-injected mice was similar to that from control mice (Mock). These results suggest that lipoplex induces the innate immune response more strongly than the Ad vector.

3.3. Hepatotoxicity caused by vector injection

The majority of intravenously injected Ad vector is sequestered by the liver, which in turn causes an inflammatory response characterized by acute transaminitis and vascular damage [7]. Systemic administration of lipoplex also leads to toxic effects in the liver [13]. We investigated the hepatic damage induced by the intravenous administration of the Ad vector and lipoplex. The hepatotoxicity induced by the vectors was compared by measuring the activity of alanine aminotransferase (ALT) in serum and by investigating the histopathological changes. There were no significant differences in the ALT activity between the sera from the Ad vector-injected mice (5×10^{10} VP/mouse) and the lipoplex-injected mice (both 5 and 25 μg plasmid-DNA/mouse) after 24 h post-injection (Fig. 4). The serum ALT activity in mice injected with 1×10^{10} VP of the Ad vector was the same as in the controls (i.e., the mock-treated levels). In the case of hepatic histological changes, hepatocytes from lipoplex-injected mice (25 μg plasmid-DNA/mouse) started to granulate 3 h after the injection, even though the cells from the Ad vector-injected mice (5×10^{10} VP/mouse) seemed to be normal (Fig. 5B and E). Six hours after injection, hepatocytes from the Ad vector-injected mice started to granulate similarly to those from lipoplex-injected mice (Fig. 5C and F). At 48 h after the injection, degranulation or denucleation occurred in hepatocytes from both the Ad vector- and lipoplex-injected mice (Fig. 5D and G). These results showed that both the Ad vector and lipoplex caused hepatotoxicity, especially at high doses, and that the liver damage caused by lipoplex, such as granulation and degranulation, started earlier than in the case of the Ad vector.

3.4. Effect of unmethylated CpG motifs in plasmid DNA on cytokine production

It has been reported that bacterial DNA induces innate immune response because it has a much higher frequency of unmethylated CpG dinucleotides than mammalian DNA [18,28]. The injection of plasmid DNA, which contains fewer CpG motifs, reduces the induction of inflammatory cytokines [13,26]. We examined the production of cytokines induced by the intravenous injection of lipoplex containing plasmid DNA without CpG motifs (non-CpG lipoplex), which is completely lacking in CpG motifs. In this experiment, only the level of inflammatory cytokine was examined, because luciferase-coding cDNA without CpG motifs was not obtained. As expected, the production of IL-6 induced by non-CpG lipoplex

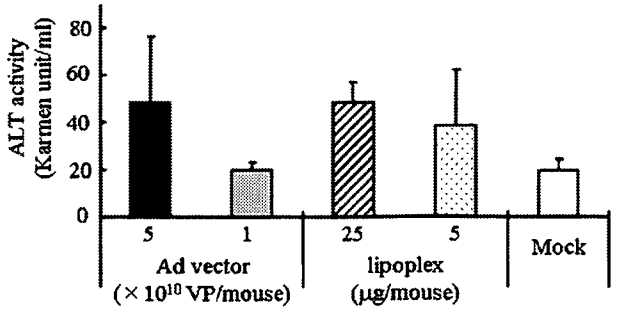

Fig. 4. Hepatotoxicity by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected intravenously into each mouse. Twenty-four hours after the injection, peripheral blood was collected, and the ALT activity was measured. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

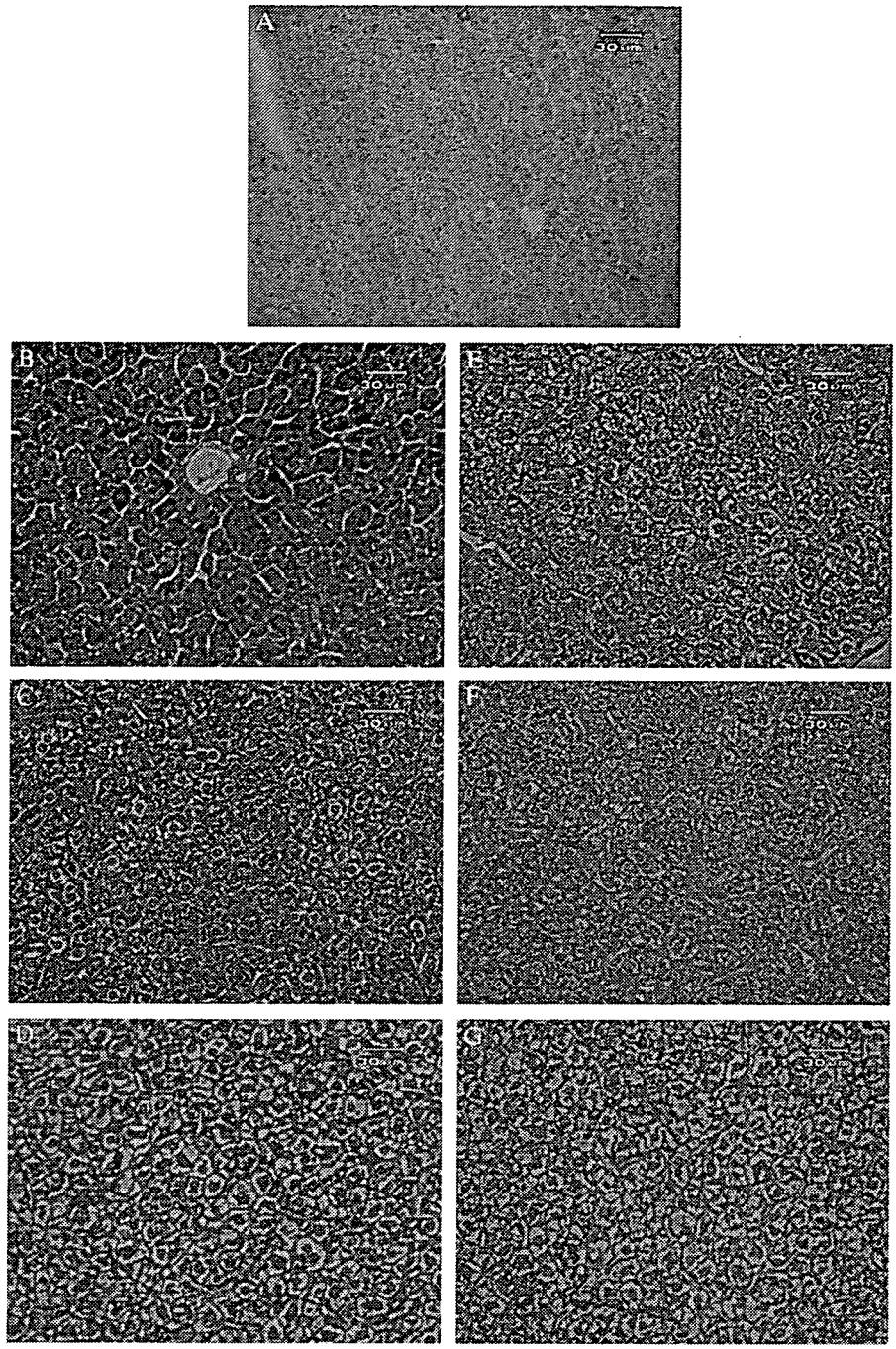

Fig. 5. Induction of acute liver failures by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse; B–D), lipoplex (25 μg of plasmid DNA; E–G) or PBS (as Mock; A) was injected intravenously into each mouse. The livers were collected after 3 (B and E), 6 (C and F), and 48 (D and G) h following the injection, and paraffin sections were prepared. Each section was stained with hematoxylin and eosin.

was suppressed, but this complex still induced higher IL-6 production than the Ad vector (Figs. 3A and 6A). The injection of non-CpG lipoplex showed a different peak time of IL-6 production than in the case of CpG lipoplex injection. The level of producton of IL-12 and TNF-α induced by the injection of non-CpG lipoplex was lower than that induced by the injection of CpG lipoplex (Figs. 3B, C, 6B and C). The concentration of IL-12 in the serum from the non-CpG lipoplex-injected mice was lower than that in the serum from the Ad vector-injected mice. Unlike in the case of IL-6, non-CpG lipoplex showed the same profiles of IL-12 production as CpG-lipoplex. These results show that the removal of CpG motifs from the plasmid DNA in lipoplex could not completely suppress cytokine production, but there exists other mechanisms for suppressing immune response by lipoplex.

4. Discussion

A variety of viral and non-viral vectors have been developed for gene therapy [3,4,10]. At present, viral vectors dominate in

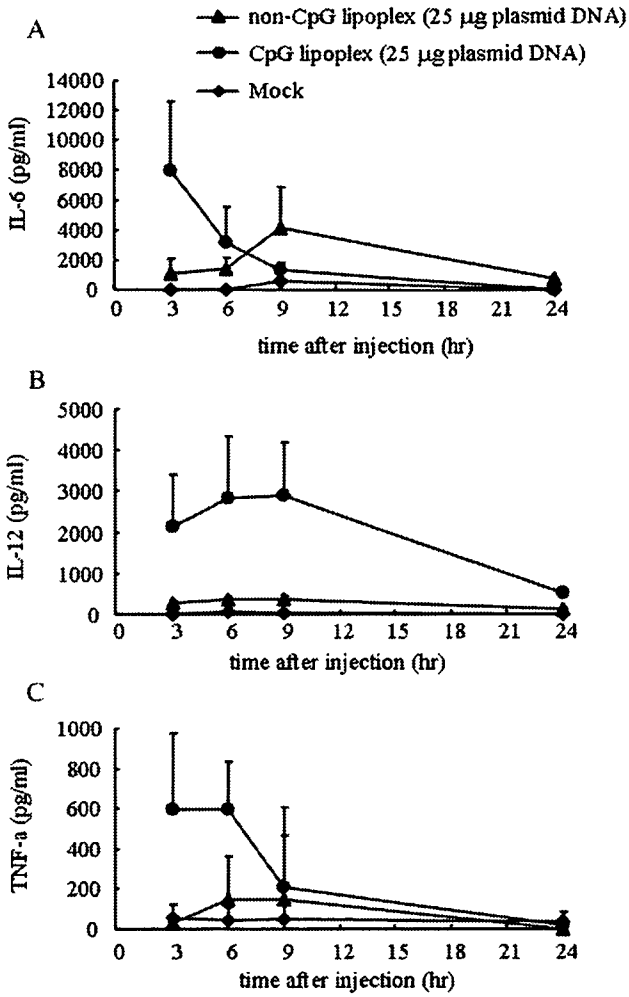

Fig. 6. Suppression of cytokine production by using non-CpG plasmid. CpG lipoplex containing pCMVL1 and non-CpG lipoplex containing pCpG-mcs were prepared as described in Material and methods. A final volume of 200 μl of CpG lipoplex (circle), non-CpG lipoplex (triangle) (25 μg of plasmid DNA) or 5% dextrose (as Mock; diamond) was injected intravenously into each mouse. Peripheral blood was collected at 3, 6, 9, 24, and 48 h after the injection, and the concentrations of IL-6 (A), IL-12 (B) and TNF-α (C) were measured by ELISA. Data are expressed as means±SD of 3–4 mice per group.

clinical trials because they are highly efficient in transducing cells. However, viral vectors are immunogenic and potentially mutagenic; thus, non-viral vectors have recently gained increasing attention [2]. In this study, we compared the in vivo usability and safety between a viral vector and a non-viral vector by examining the levels of reporter gene expression and inflammatory cytokine production (innate immune response). Our data revealed that the Ad vector, which is one of the major viral vectors used for gene therapy, is much more efficient for transduction and is also safer than lipoplex with respect to inflammatory cytokine production.

To achieve a therapeutic effect, gene therapy vectors should be able to deliver genes of interest to the designated target and to ensure their expression for an appropriate amount of time [29]. As shown in Fig. 1, the Ad vector mediated a high transduction efficiency in a wide range of organs, whereas lipoplex mediated a low level of expression only in the lung and heart. There might be two reasons for the difference in transduction efficiency between the Ad vector and lipoplex. First, the cells in most organs are composed of non-dividing cells. Ad vectors can infect both dividing and non-dividing cells, while lipoplex shows lower efficiency of gene transfer in non-dividing cells. The other reason is that there are different mechanisms for expressing transgenes between viral and non-viral vectors. The Ad vector has a unique system to be internalized into cells and to deliver foreign genes to the nucleus. Ad vectors first attach to the cell surface through an interaction between the fiber knob protein, one of the capsid proteins of Ad, and a high-affinity receptor, the coxsackievirus–adenovirus receptor (CAR). Then, another capsid protein, penton base, mediates virus internalization by receptor-mediated endocytosis. Following endosomal disruption, the partially uncoated virions traffic through the cytoplasm along microtubeles and reach the nuclear pore complex [30]. Interestingly, intravenously administered Ad vectors accumulate mainly in the liver, spleen, heart, lung and kidney in mice, even though these tissues may not necessarily have the highest level of CAR expression [29]. On the other hand, the specific receptor involved in the uptake of lipoplex remains unknown [10]. Lipoplex is taken up by an endocytosis mechanism, and the cytoplasmic delivery of DNA involves a fusion-related event, probably in the endosome compartment. One reason for the induction of limited transgene expression only in the lung and heart by lipoplex is the particle size of lipoplex, which is, on average, 350 nm, much bigger than that of the Ad vector (70–100 nm) [31,32]. Following intravenous administration, the larger lipoplex is known to lodge in the pulmonary capillaries [10,33]. The other possibility is that proteoglycans exposed at the cell surface mediate lipoplex-cell binding in the pulmonary vasculature [10,34].

For gene therapy to be successful, an appropriate amount of a therapeutic gene must be delivered into the target tissue without substantial toxicity [4]. We examined the innate immune response and hepatotoxicity induced by intravenous injection of the vectors and revealed that lipoplex was more highly immunogenic than the Ad vector, at least in terms of innate immune response. The innate immune response triggered by the Ad vector has been reported to be dose-dependent, occurs within 24 h after the injection, and is independent of viral or transgene transcription [5,30]. Our data are consistent with these observations. The production of cytokines induced by lipoplex is mainly due to the unmethylated CpG motifs in plasmid DNA [12]. Toll-like receptor 9 (TLR9) has been identified as the receptor involved in the recognition of unmethylated CpG motifs [18]. TLR9 is a member of the family of TLRs, which play a critical role in innate immunity, such as through the production of inflammatory cytokines [15,35]. Since the lipoplex is the complex of plasmid DNA and liposome, some plasmid DNAs are exposed outside the complex. When lipoplex enters the cells by endocytosis, the plasmid DNAs which are exposed outside the complex might be recognized by TLR9 expressed at the endosome. On the other hand, Ad vectors enters the cells with the genome (Ad DNA) encapsulated inside the capsid. TLR9 could not recognize Ad DNA, even though Ad DNA contains some CpG motifs, because Ad DNA is encapsulated by viral capsid in the

endosome, where TLR9 exists. A previous report showed that the absence of CpG signaling that occurs when $TLR9^{-/-}$mouse is used greatly suppresses the innate immune response induced by lipoplex, but does not completely eliminate the acute toxic response, such as cytokine production [15]. Although this is not a complete remedy, one might predict that a completely non-CpG plasmid (CpG zero plasmid) vector would have an improved safety profile. We learned that the complete removal of CpG motifs from plasmid DNA could reduce cytokine production but the levels of inflammatory cytokine production were similar to that by the Ad vector, although it depended on the type of cytokines (Figs. 3 and 6). The production of inflammatory cytokines such as IL-6 and TNF-α could not be suppressed completely, although CpG motifs were removed from plasmid DNA in lipoplex. This result suggests that there might be different pathways to induce the production of inflammatory cytokines that are activated independently of the CpG motif. Un-identified sensor receptor(s), which recognize(s) foreign DNAs, might be involved in this phenomenon [36].

In this study, we used only one kind of liposome (DOTAP/chol). We could not draw the general conclusion that all types of lipoplex would show the same profile as DOTAP/Chol in inducing the innate immune response. However, the lipoplex that enters cells using the endocytic pathway might show the same tendency, because TLR9 is expressed at the endosome. Recently, many kinds of lipoplex and polyplex (complexes of cationic polymer/plasmid DNA) have been developed for the purpose of obtaining a higher transduction efficiency and suppressing the immune response [2,37,38]. Since TLR9 expression is in the endosomes of immunocompetent cells, a non-viral vector that can escape from endosomes quickly or that is not easily taken up by immune cells should be developed to eliminate the problem of the induction of the innate immune response.

Unlike the profile of cytokine production, the activity of ALT in sera from the lipoplex-injected mice was the same as that in sera from the Ad vector-injected mice. The histopathological changes in the liver in the Ad vector-or lipoplex-injected mice also showed a similar profile to each other. The mechanism of hepatotoxicity induced by those vectors is still unclear, but inflammatory cytokines might play a role in the hepatotoxicity.

It is commonly believed that non-viral vectors are safer to use in gene therapy than viral vectors. However, this study clearly showed that this would not be true, at least in innate immune response when the vectors are systemically injected. We hope that this present study will trigger a reconsideration of the safety of gene therapy vectors.

Acknowledgments

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第Ⅰ部 医薬品開発研究の最前線

遺伝子治療研究の動向

水口 裕之*

本総説では，これまで施行されてきた遺伝子治療臨床研究と現在の国内外の研究動向，および遺伝子治療を支える基盤技術開発の現状について解説する。

■キーワード：遺伝子治療，ベクター

1 はじめに

1991年に米国において，世界で最初の遺伝子治療臨床研究がアデノシンデアミナーゼ（ADA）欠損症に対して行われてから17年が経過した。その後，遺伝子治療は先天性の遺伝性疾患だけでなく，エイズなどの感染症や癌をはじめとした後天性疾患，末梢性血管疾患をはじめとする生活習慣病に対しても広く行われ，癌で871，血管疾患で119，先天性遺伝性疾患で109，感染症で85の合計1,300以上（他の疾患に対するものを含む）のプロトコールで遺伝子治療が実施されている。（2007年7月現在）。（http://www.wiley.co.uk/genmed/clinical/）。わが国では，現在までに16のプロトコールでの遺伝子治療臨床研究が実施されている。これらの遺伝子治療臨床研究（試験）は第Ⅰ・Ⅰ/Ⅱ相試験が，合わせて全体の81%を占めており，第Ⅲ相試験は全体の2.4%にすぎない。本総説では，国内外における遺伝子治療臨床研究の動向と，遺伝子治療を成功に導くために必要な基盤技術開発の現状について解説する。

2 遺伝子治療の成功例

遺伝子治療臨床研究における代表的な成功例としては，ADA欠損症と重症複合免疫不全症（X-SCID）があげられる。ADAはデオキシアデノシンあるいはアデノシンを，イノシンもしくはデオキシイノシンに変換するための触媒酵素であり，本酵素の欠損によりアデノシン，デオキシアデノシンが蓄積すると，DNA複製・修復機構に傷害が生じ，リンパ球減少を特徴とする複合免疫不全症となる。1990年代に行われたADA欠損症に対する遺伝子治療では，末梢血中のリンパ球にADAをつくる正常な遺伝子をレトロウイルスベクターを用いて導入した後，患者に戻す治療が行われ，酵素補充療法（PEG-ADA：ポリエチレングリコール〔PEG〕修飾されたADA酵素の投与）との併用として施行された。わが国においては北海道大学で同様の治療が行われ，治療を受けた患者は日常生活を送られる程の回復がみられている。その後，末梢血中のリンパ球にADA遺伝子を導入するプロトコールでは，細胞寿命の関係で定期的な遺伝子治療を受ける必要があることから，造血幹細胞（CD34陽性細胞）にADA遺伝子を導入するプロトコールが世界的に行われた。特筆すべきは，北海道大学で行われた造血幹細胞を標的としたADA遺伝子治療では，世界で初めてPEG-ADAを併用せずに免疫機能の回復が認められたことで

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あり，現在患者は有害事象もなく普通に学校に通っている。

X-SCIDはインターロイキン受容体の一部として共用されているγc鎖の変異によって発症する先天性の免疫不全症であり，重症感染症により幼児期までに死亡する致死的疾患である。フランスで行われたX-SCIDに対する遺伝子治療では，CD34陽性細胞にγc鎖遺伝子をレトロウイルスベクターを用いて導入し，10例中9例で著効（1例は感染にて不生着）を示し，治療2～3カ月後には多種抗原に対するT細胞免疫機能が回復した。（B，NK細胞も部分回復）[1,2]。生存することが困難な遺伝病から劇的な回復を見せた治療法であり，世界的に大きな注目を集めたが，後述するように治療2～3年後に計3例の患者で白血病が発症するという有害事象が起こり（2007年になってから，4例目の白血病発症が報告されている），先端医療研究全体に対して極めて強い衝撃を与えた。

その他，慢性肉芽腫症（活性酸素生産酵素であるNADPH oxidaseとよばれる酵素複合体を形成する分子の欠損により，好中球が細菌やカビを殺傷する活性酸素を作ることができず，重篤な微生物感染を繰り返す遺伝性疾患）に対する遺伝子治療においても症状の改善が認められている[3]。

3 遺伝子治療における有害事象

先端医療である遺伝子治療を推進していく上では，安全面には特段の注意が必要である。しかしながらこれまでに，遺伝子治療における有害事象の代表的なものとして，アデノウイルスベクター投与に伴う死亡事故と，レトロウイルスベクターによる染色体への治療用遺伝子の挿入に伴う白血病の発症が報告されている。

1999年，米国ペンシルバニア大学でオルニチントランスカルバミラーゼ（OTC）欠損症（OTC遺伝子の異常により，肝臓でアンモニアを分解できない遺伝病）の遺伝子治療臨床研究の経過中，大量のアデノウイルスベクターの全身投与によって，一人の男性患者が4日後に多臓器不全で死亡するという事故が起こった。本遺伝子治療は肝臓の血管内にベクターを投与するプロトコールにより行われたが，大量に（プロトコール中最大の投与量）投与されたアデノウイルスベクターにより免疫反応の過剰な活性化が起こり，全身性炎症反応症候群（systemic inflammatory response syndrome：SIRC）を生じたことが原因と考えられている。この事故は，プロトコールの遂行や管理が適切でなかったこともあり，大きな社会問題となった。これまでにアデノウイルスベクターを血管内に投与するプロトコールによる臨床研究は行われてきたが，この死亡した患者ほどの免疫反応を生じた例はなく，アデノウイルスベクターにより誘発される免疫反応の程度は患者毎に大きく異なることが示唆される。今後，アデノウイルスベクターにより生じる免疫反応の詳細なメカニズム解明とその制御法の開発，および重度の免疫反応を誘発する原因（予測因子）を明らかにすることが必要となるであろう。

X-SCID遺伝子治療における白血病の発症は，治療後，2～3年を経過した後に起こった[4]。白血病を発症した3例の患者とも，γc鎖遺伝子がプロトオンコジーンのLMO2遺伝子の近傍に挿入され，LMO2遺伝子の活性化によりモノクローンのT細胞が増殖優位性を獲得したことが白血病化の原因と考えられている[5]。造血幹細胞（CD34陽性細胞）を用いた遺伝子治療は他の疾病に対しても行われているが，白血病の発症は報告されておらず，X-SCIDという特異な疾病と，様々な要因が複合的に絡み合って白血病化に至ったことが推測される。一方で，この事故を経緯に，染色体への遺伝子挿入部位の分析法が新たな基盤技術として確立され，他症例を含むレトロウイルスベクターを用いた遺伝子治療において，遺伝子挿入部位の解析とモニタリング強化が対策として取られている。

また，2007年7月末に，米国において，アデノ随伴ウイルスベクターを用いてTNF-α（tumor necrosis factor-α）レセプターを治療用遺伝子としたリウマチに対する遺伝子治療で死亡例が報告されたが，現在のところ遺伝子治療と死亡との直接的な因果関係の有無も含め，詳細な原因は発表されていない。

4 国外における遺伝子治療臨床研究の動向

1．癌を対象にした研究動向

2003年には，中国のSiBiono社が世界初の遺伝子治療医薬品であるGendicine（p53を発現するアデノウイルスベクター）を頭頸部扁平上皮癌に対する製剤として認可され，中国においてはすでに多数の患者が遺伝子治療医薬品の投与を受けている[6]。p53を発現するアデノウイルスベクター（Advexin®，Introgen Therapeutics社）は米国においてもファーストトラック（画期的な新薬について優先的に審査する制度）に指定され，審査中である。Vical社（米国）はステージⅢもしくはⅣの再発進行期メラノーマに対して，MHC（major histocompatibility complex）-1もしくはMHC-1抗原を形成するHLA-B7とβ2ミクログロブリンをコードするプラスミドのカチオニックリポソーム製剤（Allovectin-7：腫瘍内投与により局所および転移癌に対する免疫刺激作用が期待できる）の第Ⅲ臨床試験を行っており，FDAからオーファンドラッグに指定されている。

癌に対する遺伝子治療においては，サイトカインやケモカインを発現するベクターを腫瘍内投与したり，これらを産生する細胞を移植したりすることでワクチン的な治療効果を期待するプロトコールや，p53などの癌抑制遺伝子や自殺遺伝子（ヘルペスウイルス由来のチミジンキナーゼなど）を発現するベクターを腫瘍内に投与するプロトコールによる治療が当初盛んに試みられてきた。しかしながら，遺伝子治療適用患者が既存の治療法では治療効果が得られない末期患者が中心であることも原因であるが，優れた治療効果を示すことは少ないのが現状である。そこで，より積極的に癌を死滅させるアプローチとして，癌細胞でだけ特異的に複製する組換えウイルス（アデノウイルスやヘルペスウイルスが多く用いられる）を用いたウイルス療法が近年盛んに行われている。例えば，Onyx社（米国）が開発を進めているONYX-015は，アデノウイルスの複製に必須のE1Bによってコードされる55キロダルトンのタンパク質を欠損させたウイルス（dl1520）であり，p53を欠損した細胞（多くの癌細胞）で特異的に複製し細胞を殺傷する[7]。ONYX-015は多施設での臨床試験が進められたが，同社は現在開発を中止している。ちなみに，中国ではONYX-015とほぼ同等のウイルス製剤であるH101がすでに医薬品として承認されている。また，腫瘍特異的なプロモーターでアデノウイルスの複製に必須のE1A（とE1B）遺伝子を発現させることで，腫瘍特異的にウイルス複製を生じさせ，細胞を死滅させるアプローチも多数試みられている。一方，ヘルペスウイルスについても，病原性に関連したウイルス遺伝子のγ34.5遺伝子やICP6遺伝子に変異を入れたウイルス（G207）を用いた治療が，脳腫瘍等を対象に行われ，現在更なる改変を加えた組換えヘルペスウイルスの開発が進んでいる。他に，ワクシニアウイルスや麻疹ウイルスを用いたウイルス療法による臨床試験も行われている。

2．その他の疾患を対象にした研究動向

血友病B（血液凝固第Ⅸ因子の欠損による出血性疾患）に対するアデノ随伴ウイルス（adeno-associated virus：AAV）ベクターを用いた遺伝子治療が米国のHighおよびKayらのグループで積極的に進められている。血友病は正常レベルの1～2％の第Ⅸ因子が産生されれば臨床（治療）効果が期待できることから，遺伝子治療の理想的な対象疾患と考えられている。2型AAVベクターを用いた血友病Bに対する前臨床試験では，ベクターを門脈内から投与し，肝臓で第Ⅸ因子を発現させることでマウスおよび血友病Bのモデルイヌにおいて治療レベルの第Ⅸ因子の発現が長期間（1～2年以上）続く良好な結果が得られていたため[8]，臨床研究での成功も有望視されていた。当初の臨床研究はより安全性の高いと考えられる骨格筋を標的として行われたが，有効なレベルの凝固因子の血中濃度上昇が認められた例は少なかった[9][10]。そこで本来の第Ⅸ因子産生臓器である肝臓を標的とした治療が行われ，治療レベルの凝固因子の産生が見られたが，動物実験の場合とは異なり，効果は一過性であった[11]。その原因について解析したところ，投与されたAAVベクター（中のカプシドタンパク質）が肝細胞に外来性抗原として抗原提示され，遺伝子導入細胞が免疫系に排除されて

いることが明らかとなった。動物実験では（おそらく提示された抗原が免疫反応を惹起するレベルにまで達していなかったため）遺伝子導入細胞の排除は認められなかったが，ヒトではより高感度にこのような免疫反応を起こすことが示され，より低容量のベクター投与でも治療効果が期待できる新たなAAVベクターの開発，あるいはベクター投与時に免疫抑制剤で一時的に免疫を抑制する必要性が考えられた。なお，血友病患者の場合，産生された凝固因子そのものが異物と認識される可能性もあるが，幸運にも抗凝固因子抗体の産生は認められなかった。現在，免疫抑制剤との併用による遺伝子治療臨床研究の準備が進められている。

パーキンソン病に対しては，主にAAVベクターを用いて，様々な遺伝子（アプローチ）で臨床研究が進められている。芳香族アミノ酸脱炭酸酵素（AADC：L-DOPAをドパミンに変換する酵素）を線条体に発現させ，L-DOPAの内服と組み合わせて効果を期待するアプローチは米国で既に臨床研究が進められており，わが国でも2007年，自治医科大学が同様のアプローチによる遺伝子治療を開始した。また，米国コーネル大学のグループは最近，神経細胞の活動を抑制することを目的にGAD（glutamic acid decarboxylase；抑制性神経伝達物質のGABA（γ-アミノ酪酸）の産生に必要な酵素）遺伝子を投与するアプローチで，治療3カ月後より治療効果が認められ，少なくとも1年間の病状の改善が認められたことを報告している[10]。

最近，Cardium Therapeutics社（米国）が開発を進めている血管新生促進遺伝子治療製剤Generx（FGF-4［Fibroblast Growth Factor-4］を発現するアデノウイルスベクター）が冠動脈疾患による再発性狭心症を対象として米国においてファーストトラック指定を受け，医薬品化が期待されている。

5 国内における遺伝子治療臨床研究の動向

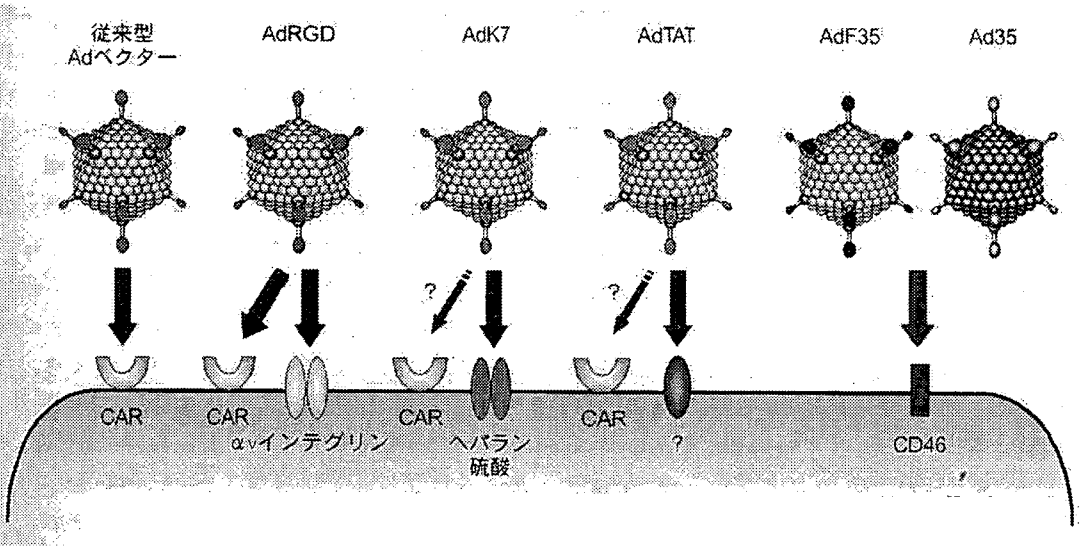
わが国における遺伝子治療臨床研究は，欧米と比較するとかなり遅れているのが実情であるが，最近の国内における遺伝子治療臨床研究（試験）の特徴としては，ベンチャー企業などが先導して臨床研究（試験）を展開していることである。大阪大学発のベンチャーであるアンジェスMG社は，末梢性血管疾患（閉塞性動脈硬化症）に対してHGF（Hepatocyte growth factor）発現naked DNAを筋肉内に注射することで血管新生を誘導する臨床試験を進め，第III相試験でプラセボ群と比べ有効性に顕著な差を認めている。現在承認申請に向けた準備を進めており，初の『"目の丸"遺伝子治療医薬品』となることが期待される。岡山大学発のベンチャーであるオンコリスバイオファーマ社は，癌細胞特異的なプロモーターを利用してE1A，E1Bタンパク質を発現させることにより癌細胞特異的に複製可能なアデノウイルス製剤（telomelysin）の開発を進めており，2006年末に米国において進行性の乳ガン患者に対する第I相試験を開始した。大阪大学発のベンチャーであるクリングルファーマ社は，血管新生阻害活性を有するNK4を発現するアデノウイルスベクターを用いた癌に対する遺伝子治療臨床試験を計画中である。神戸大学発のベンチャーであるジーンメディスンジャパン社は，癌細胞特異的に複製するアデノウイルスを産生するキャリア細胞を利用した癌に対する臨床試験を米国および中国で計画中である。同社は，遺伝子治療用ベクターの製造および細胞治療用セルプロセッシングをGMP（good manufacturing practice）準拠にて行うことが可能な施設を備えており，ウイルスベクター製品の受託製造を行っていることも大きな特徴である。ディナベック社は，独自開発したセンダイウイルスベクターを用いて重症虚血肢に対してFGF-2を発現させ，血管新生を誘導する遺伝子治療を2006年九州大学で開始している。同社は中国のSiBiono社とエイズワクチン開発に関する契約を締結しており，わが国で開発された技術が海外にも導出されている。タカラバイオ社は，韓国と中国で関連会社などを通して，それぞれ慢性肉芽腫症および癌に対する遺伝子治療の計画を進めている。

6 遺伝子治療を支える基盤技術の開発動向

これまで行われてきた遺伝子治療臨床研究は，主に1990年代に開発された第一世代のベクターが主に用いられてきたが，臨床研究での効果が十分ではないことや，有害事象の発生などもあって，より安全性および有効性の高いベクターの開発や，遺伝子治療を支える周辺基盤技術の開発が積極的に進められている。以下に，各種ベクター系開発の最近の進歩について簡単に解説する。

1．アデノウイルスベクター

従来のアデノウイルスベクターはヒト5型アデノウイルスを基盤としたものであるが，本ベクターは，①遺伝子導入がアデノウイルス受容体(coxsackievirus and adenovirus receptor：CAR)の発現に依存し，CARの発現がない細胞(特に悪性度の高い癌細胞や血液系細胞等)へは遺伝子導入できないこと，②免疫反応を伴うこと，などの問題点があった。①に関しては，細胞との結合を担うウイルスカプシドタンパク質のファイバー表面上に，特定の細胞受容体との結合を担うペプチド配列を挿入したり，ファイバー部分や全てのウイルスタンパク質をCAR以外の分子(ヒト由来細胞ではほぼ全ての細胞に発現が認められるCD46など)を認識する他のアデノウイルス由来のものに置換したりしたベクターが開発されている。(**図1**)[13]。②に関しては，ウイルスコード遺伝子を全て除去したgutted アデノウイルスベクターを用いてウイルスタンパク質の産生を欠失させたベクターや[14]，ファイバー領域を変更することで*in vivo*投与後の免疫担当細胞への感染を減弱させたベクターが開発されている[15]。また，gutted アデノウイルスベクターに，Sleeping Beauty[16]やバクテリオファージインテグラーゼΦC31[17]，レトロトランスポゾン[18]の染色体への遺伝子組み込み活性を付与することで，導入遺伝子が積極的に染色体に組み込む活性をもたせたアデノウイルスベクターの開発も進められており，



■図1　各種改良型アデノウイルスベクター

野生型のファイバーを持った従来型の5型アデノウイルスベクターは細胞表面上の受容体であるCARを認識して感染するが，RGD配列やポリリジン配列をファイバーに有したファイバー改変ベクター(AdRGD，AdK7)はCARだけでなくαvインテグリンやヘパラン硫酸を認識しても感染できる。TATペプチドを付与したAdTATは，詳細な細胞内移行メカニズムは不明であるが，CAR非依存的に感染できる。また，35型のアデノウイルスのファイバーを有したベクター(AdF35)や，全ての構造タンパク質が35型アデノウイルスからなるベクター(Ad35)は，CD46を認識して感染する。アデノ随伴ウイルスベクターにおいても，アデノウイルスベクターと同様のカプシド改変ベクターや血清型を変更したベクターの開発が進んでいる。