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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 $\times g$ 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

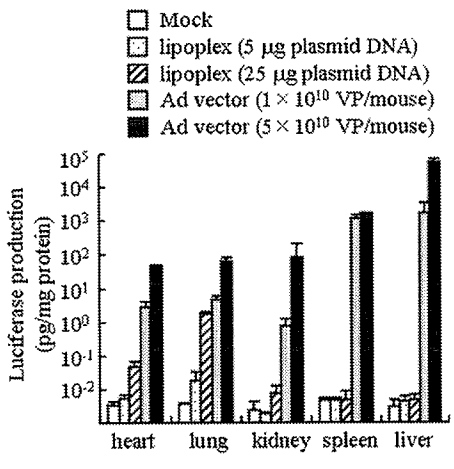

Fig. 1. Luciferase production in various organs after intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; dotted and slashed bar, respectively) was injected 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.

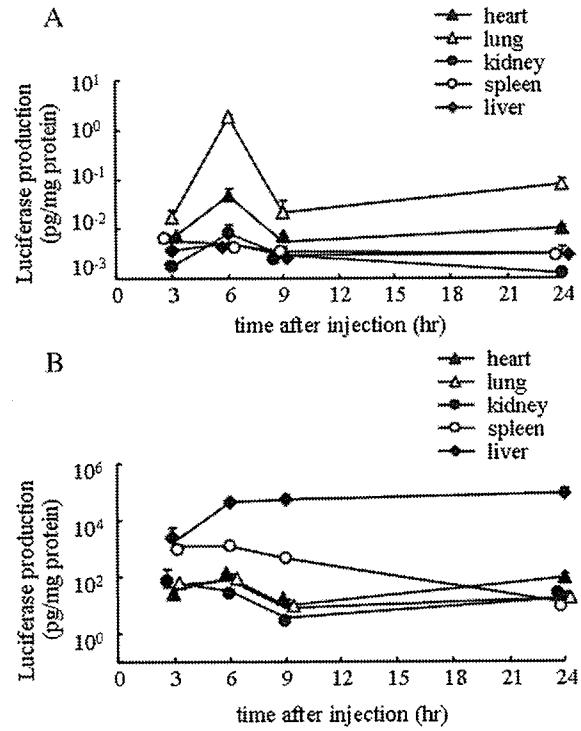

Fig. 2. Duration of the luciferase production. A final volume of 200 μl of lipoplex (25 μg of plasmid DNA; A) or Ad vectors (5×10^{10} VP/mouse; B) was injected intravenously into each mouse. After an appropriate length of time had passed following the injection, the heart (filled triangle), lung (opened 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

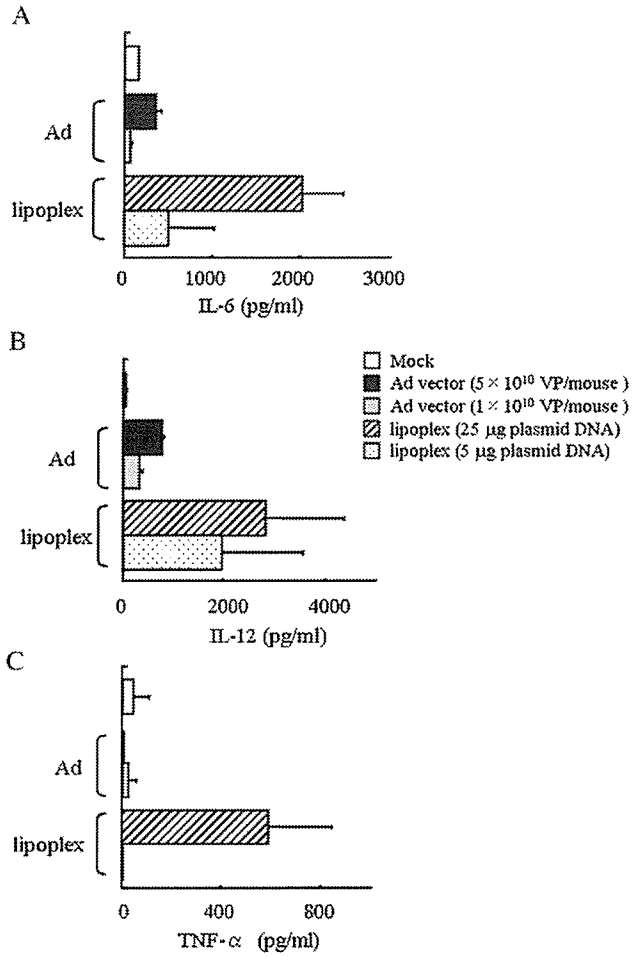

Fig. 3. Induction of various inflammatory cytokines by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected 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

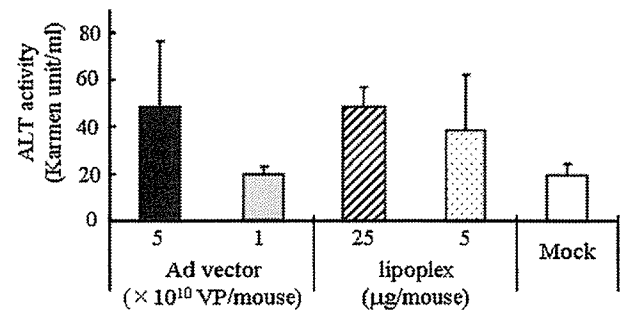

Fig. 4. Hepatotoxicity by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected intravenously into each mouse. 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.

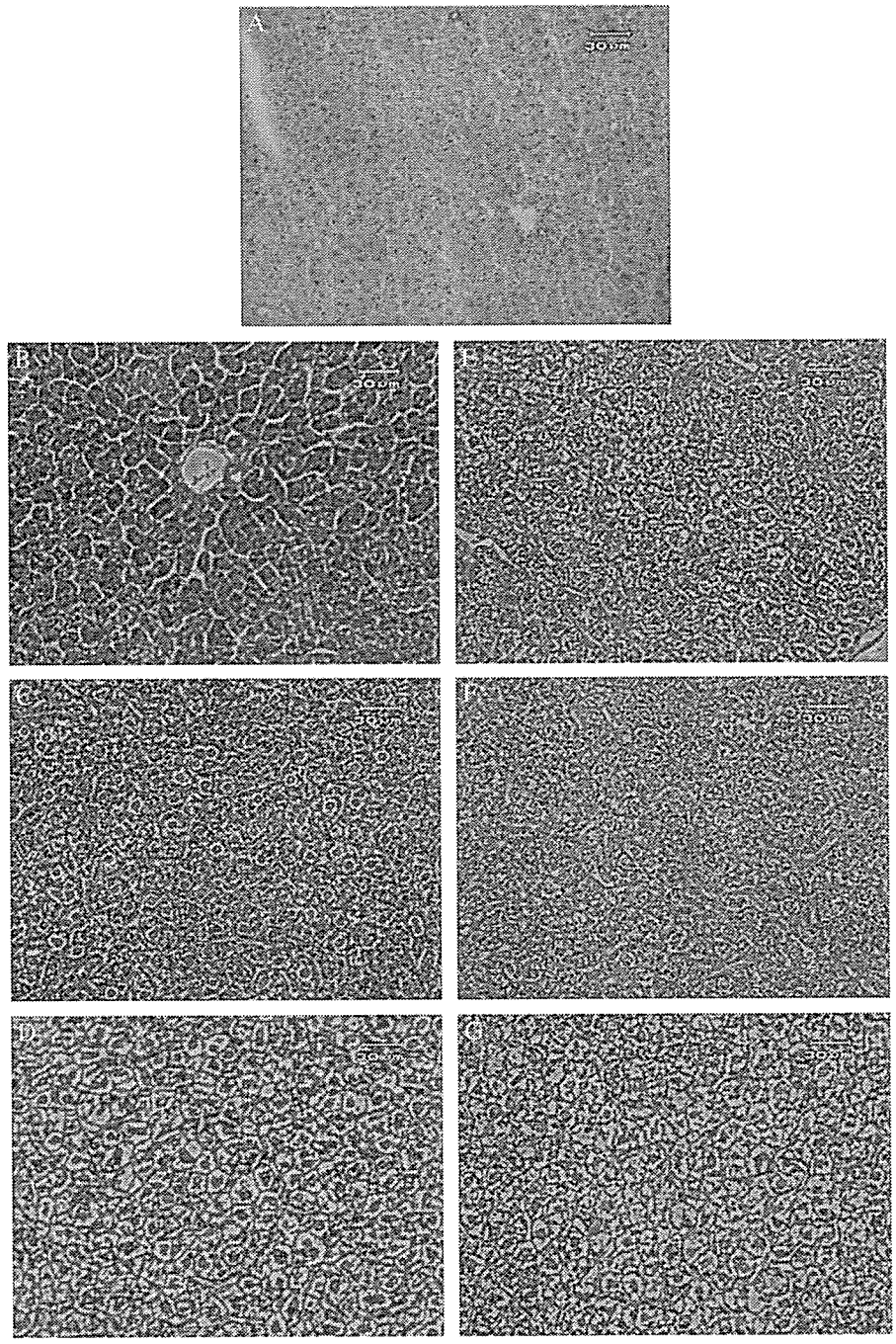

Fig. 5. Induction of acute liver failures by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse; B–D), lipoplex (25 μg of plasmid DNA; E–G) or PBS (as Mock; A) was injected intravenously into each mouse. The livers were collected after 3 (B 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

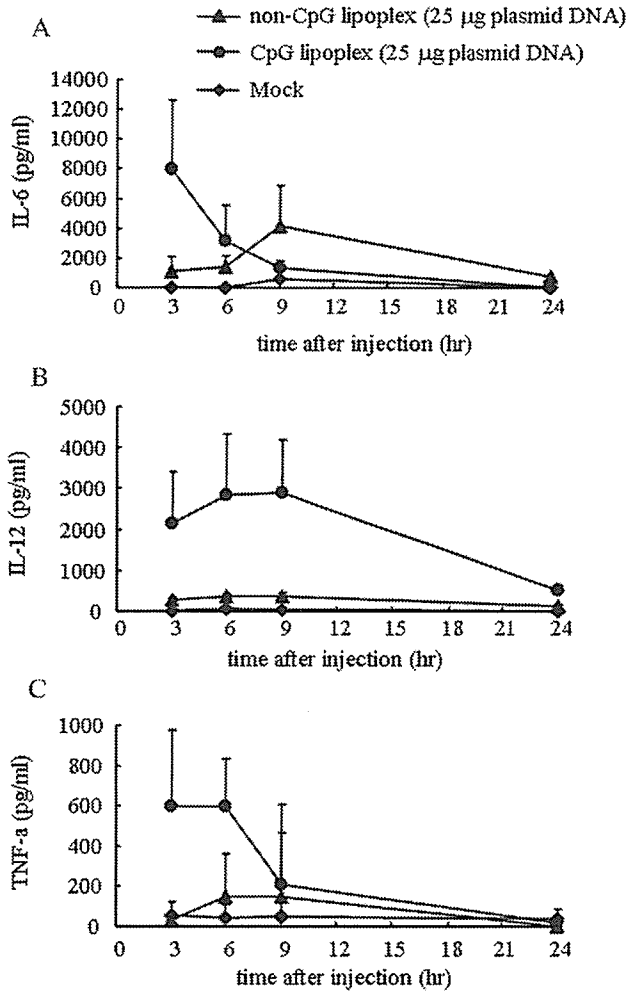

Fig. 6. Suppression of cytokine production by using non-CpG plasmid. CpG lipoplex containing pCMVL1 and non-CpG lipoplex containing pCpG-mcs were prepared as described in Material and methods. A final volume of 200 μl of CpG lipoplex (circle), non-CpG lipoplex (triangle) (25 μg of plasmid DNA) or 5% 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.

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Role of Tyr306 in the C-terminal fragment of *Clostridium perfringens* enterotoxin for modulation of tight junction

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ABSTRACT

We previously reported that the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) is a novel type of absorption enhancer that interacts with claudin-4 and that Tyr306 of C-CPE plays a role in ability of C-CPE to modulate barrier of tight junctions. In the current study, to investigate effects of Tyr306 on the C-CPE activity, we prepared some C-CPE mutants substituted Tyr306 with Trp (Y306W), Phe (Y306F) and Lys (Y306K). We found that Y306W and Y306F mutants of C-CPE had claudin-4 binding affinities and effects on the barrier function of tight junctions, whereas both of these properties were greatly reduced with the Y306K mutant. Finally, the Y306K but not the Y306F and Y306W mutants had reduced abilities to enhance absorption in rat jejunum. These results indicate that aromatic and hydrophobic properties, not hydrogen bonding potential, of Tyr306 are involved in the interaction of C-CPE with claudin-4 and in the modulation of the tight junction barrier function by C-CPE.

1. Introduction

Recent dramatic progress in drug design based on genomic data has produced a large number of leads that must be analyzed by high-throughput screening, but delays in the development of drug delivery has slowed their clinical testing and application. Drugs can be delivered to their target tissues by two pathways: transcellular and paracellular. The paracellular route is thought to be the most reasonable for hydrophilic macromolecular drugs, such as peptides and

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Abbreviations: C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; PSIF, protein synthesis inhibitory factor; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; TER, transepithelial electric resistance; C-CPE-PSIF, C-CPE fused to PSIF; PCR, polymerase chain reaction; LDH, lactate dehydrogenase; FD-4, fluorescein-isothiocyanate-dextran with a molecular weight of 4000

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proteins, obtained by high-throughput screening because delivery through the transcellular route would require modification of each drug for recognition by specific transporters.

Tight junctions (TJs) between cells seal the paracellular route, preventing leakage of substances [1]. The TJs are complex biochemical systems in which claudins, transmembrane proteins with four membrane-spanning domains, play a pivotal role [2]. There are 24 members of the claudin family, each of which has different expression profiles and barrier functions. For instance, claudin-1 and claudin-5 contribute to the barrier functions of the epidermis and the blood–brain-barrier, respectively [3,4]. Tsukita and Furuse proposed that claudins function as a barrier by forming homo- and/or hetero-dimers [2] and that the many possible patterns of dimerization can explain the diversity in TJ barrier function [2,5,6]. These findings indicate that claudins could be targeted for the development of drug delivery systems.

Clostridium perfringens enterotoxin (CPE) causes the symptoms associated with *C. perfringens* food poisoning in humans [7]. CPE has two functional domains: an N-terminal cytotoxic region and a C-terminal binding region (C-CPE) [7]. C-CPE is involved in the interaction between CPE and claudin-3 or claudin-4 [8,9]. Interestingly, C-CPE modulates the barrier function of TJs in epithelial cell lines [9]. Therefore, using C-CPE as a claudin modulator, we previously investigated claudin as a target for the enhancement of drug delivery. We found that C-CPE was over 400-fold more potent at enhancing absorption than sodium caprate, an enhancer of drug absorption that is clinically used in Japan, Denmark, and Sweden [10]. Identification of the domain in C-CPE that mediates this effect should help in the development of modulators of other claudins. So far, we have narrowed the functional region of C-CPE mediating claudin-4 binding to the C-terminal 16 amino acids [10,11]. Very recently, we found that three tyrosine residues in the C-terminal 16 amino acids at position 306, 310, and 312 are responsible for the modulation of TJ barrier function by C-CPE, and Tyr306 is the most important residue among them [12]. In the current study, we try to address the reason why Tyr306 are involved in abilities of C-CPE to bind to claudin-4 and modulate TJ barrier function by site-directed mutagenesis.

2. Materials and methods

2.1. Materials

Anti-His-tag antibody and anti-claudin-4 antibody were obtained from Novagen (Madison, WI) and Zymed Laboratories (South San Francisco, CA), respectively. Ni-agarose resin was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell cultures

The human intestinal cell line Caco-2 was cultured in Dulbecoo's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C. Passages 65–72 were used for experiments. Claudin-4-expressing mouse fibroblast L cells (CL4/L cells) were kindly provided by Drs. S. Tsukita and M. Furuse [9,13]. CL4/L cells were maintained in modified Eagle's medium containing 10% fetal bovine serum at 37 °C.

Table 1 – Primers used for site-directed mutagenesis

Primers	Sequences (5′ to 3′)
Common forward primer	ggaattc cat atg gaa aga tgt gtt tta aca gtt cca tct aca
Reverse primer for Y306A	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *agc* tga tga att agc ttt cat tac
Reverse primer for Y306F	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *aaa* tga tga att agc ttt cat tac
Reverse primer for Y306W	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *cca* tga tga att agc ttt cat tac
Reverse primer for Y306K	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *ctt* tga tga att agc ttt cat tac

The underline in forward primer and in reverse primer is *NdeI* site and *BamHI* site, respectively. The italic letters in the reverse primer indicated the site of mutation.

2.3. Preparation of mutated C-CPEs

The indicated residues were mutated by polymerase chain reaction (PCR) using a forward primer containing *NdeI* site, a reverse primer containing a *BamHI* site, and pET16bHiS_{10}C-CPE as a template [10]. The primer sequences are listed in Table 1. The resulting PCR products were ligated with *NdeI*/*BamHI*-digested pET16b vector (Novagen), and the DNA sequence was confirmed. Each plasmid was transduced into *Escherichia coli* BL21 (DE3), and production of mutant C-CPEs were induced by addition of isopropyl-β-D-thiogalactopyranoside. The cells were harvested and lysed in buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, and 1 mM β-mercaptoethanol) containing 8 M urea. The lysates were applied onto a Ni-NTA column, and mutant C-CPEs were eluted with buffer A containing 100–1000 mM imidazole. The buffer was exchanged with phosphate-buffered saline by gel filtration using a PD-10 column (GE Healthcare Bio-Sciences Co., Piscataway, NJ). The concentrations of mutant C-CPEs were estimated using a protein assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules, CA). The purification of mutant C-CPEs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining of the gels with Coomassie Brilliant Blue (data not shown).

2.4. Pull-down assay

Confluent Caco-2 cells were harvested and lysed in lysis buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, and 1% protease inhibitor cocktail [Sigma–Aldrich, St. Louis, MO]). C-CPE or mutant C-CPEs were incubated with the lysates for 30 min at 37 °C, after which Ni-agarose resin was added. After an additional 3-h incubation at 4 °C, the resin was washed with lysis buffer. The proteins

bound to the resin was separated by SDS-PAGE and analyzed by Western blotting using anti-human claudin-4 and anti-His tag antibodies. Bound primary antibodies were detected using a peroxidase-labeled secondary antibody and chemiluminescence reagents (GE Healthcare Bio-Sciences Co.).

2.5. Preparation of mutant C-CPE-PSIF

The plasmids expressing mutant C-CPE fused to protein synthesis inhibitory factor (C-CPE-PSIF) were prepared as follows. PSIF is an approximately 40 kDa fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa* (ATCC strain No. 29260). We cloned the cDNA for PSIF from *P. aeruginosa*, *Migula* by PCR using the primer set 5′-gat gat cga tcg cgg ccg cag gtg cgc cgg tgc cgt atc cgg atc cgc tgg aac cgc gtg ccg cag act aca aag acg acg acg aca aac ccg agg gcg gca gcc tgg ccg cgc tga cc-3′ and 5′-gat cga tcg atc act agt cta cag ttc gtc ttt ctt cag gtc ctc gcg cgg cgg ttt gcc ggg-3′. C-CPE fragments containing mutations of Tyr306 to Ala, Phe, Trp, and Lys and NcoI and NotI sites at the 5′- and 3′-ends, respectively, were amplified by PCR using a common forward primer (5′-cat gcc atg gcc gaa aga tgt gtt tta aca gtt cc-3′; NcoI site underlined), a common reverse primer (5′-ata gtt tag cgg ccg caa att ttt gaa ata ata ttg aat aag g-3′; NotI site underlined), and a pET16b plasmid containing mutant C-CPE as a template. The NcoI/NotI-digested mutant C-CPE fragments were inserted into NcoI/NotI-digested pY02-C-CPE-PSIF to generate pY02-Y306A-PSIF, pYO2-Y306F-PSIF, pYO2-Y306W-PSIF, and pYO2-Y306K-PSIF plasmids [14]. The sequence of the plasmids was confirmed. The C-CPE-PSIF and mutant C-CPE-PSIF plasmids were transduced into *E. coli* strain TG1. The cells were grown at 37 °C in 2YT medium containing 2% glucose to an optical density at 600 nm of 0.6–0.9, and the medium was changed to 2YT medium containing 1 mM isopropyl β-D-thiogalactopyranoside. After an additional 18-h of culture at 30 °C, the conditioned medium was collected and applied to an anti-FLAG M2 affinity column (Sigma–Aldrich). Bound proteins were eluted with FLAG peptide (Sigma–Aldrich). The buffer was exchanged with phosphate-buffered saline using a PD-10 column. Purification of mutant C-CPE-PSIF proteins was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-FLAG M2 antibody (data not shown). Protein levels were determined using a commercially available assay kit with bovine serum albumin as a standard (Bio-Rad).

2.6. C-CPE-PSIF-induced cytotoxicity

CL4/L cells were pretreated with C-CPE or mutant C-CPE at the indicated concentration for 1 h, and C-CPE-PSIF was added to the cells. After an additional 36 h of culture, the release of lactate dehydrogenase (LDH) from cells was assayed. CL4/L cells were treated with C-CPE-PSIF or mutant C-CPE-PSIF at the indicated concentration for 36 h. Next, the LDH release was measured using a CytoTox96 Non Radioactive Cytotoxicity Assay kit according to the manufacturer's protocol (Promega, Madison, WI). LDH release was calculated using the following equation: maximal LDH release (%) = 100 × (LDH in the cultured medium/total LDH in the culture dish).

Cytotoxicity of C-CPE-PSIF and mutant C-CPEs-PSIF was evaluated by measuring LDH release as described above.

2.7. TER assay

Caco-2 cells were seeded in Transwell chambers (6.5-mm diameter, 0.03 cm^2 area, 0.45-μm pore diameter; Nunc, Roskilde, Denmark) at a subconfluent density. The formation of TJ barriers in Caco-2 monolayers was monitored by measuring transepithelial electric resistance (TER) using a Millicell-ERS epithelial volt-ohmmeter (Millipore Corporation, Billerica, MA). When the TER values reached a plateau, Caco-2 monolayer cells were treated with C-CPE or mutant C-CPEs on the basal side of the chamber, and the TER values were measured. Treatment of the cells with C-CPE on the apical side did not disrupt TJ barriers (data not shown). Similar results were found in cytotoxic assay of CPE in Caco-2 cells [7]. The TER values were multiplied by the area of the Caco-2 monolayer. The TER value of a blank Transwell chamber was subtracted from the TER of cell monolayers.

2.8. In situ loop assay

Wistar male rats (250–280 g) were obtained from Animal and Material Laboratories, Inc. (Tokyo, Japan). The rats were maintained in an environmentally controlled room (23 ± 1.5 °C) with a 12-h light/12-h dark cycle and allowed access to standard rodent chow and water ad libitum. The rats were allowed to adapt for a week. The experimental protocol for the in situ loop assay was approved by the ethics committee of Showa Pharmaceutical University. Intestinal absorption of fluorescein-isothiocyanate-dextran with a molecular weight of 4000 (FD-4) was investigated by in situ loop assay as follows. Rats were anesthetized with thiamylal sodium (Mitsubishi Pharma Co. Ltd., Osaka, Japan). A midline abdominal incision was made, and the lumen of the jejunum was washed with saline. A jejunal loop (5 cm in length) was prepared by closing both ends with sutures. A mixture of FD-4 and C-CPE proteins in 200 μl of PBS was administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma concentration of FD-4 was determined with a fluorescence spectrophotometer (Fluoroskan Ascent FL; Thermo Electron Corp., Waltham, MA). The area under the plasma concentration–time curve from 0 to 4 h (AUC_{0-4}) was calculated by the trapezoidal method.

2.9. Statistical analysis

Statistical significance of differences was assessed using one-way analysis of variance followed by Dunnett's test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Interaction of Y306F, Y306W, and Y306K mutants with claudin-4

We previously found that C-CPE modulates the TJ barrier and interacts with claudin-4 through its C-terminal 16

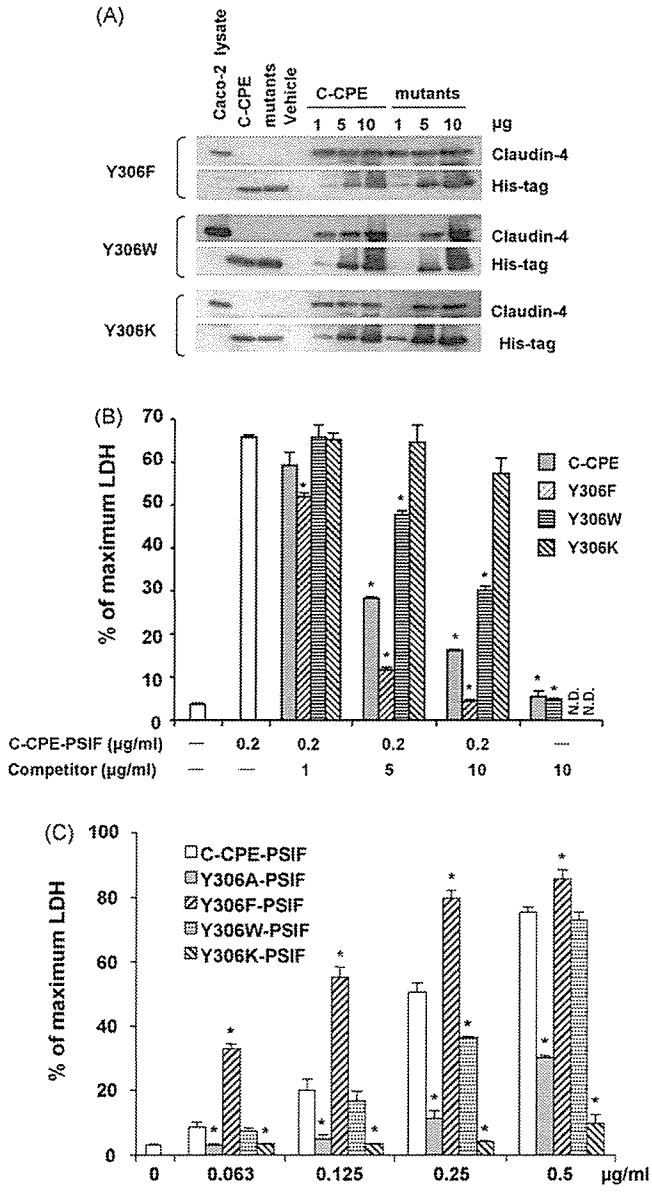

Fig. 1 – Effects of mutation of Tyr306 to Phe, Trp, and Lys on the interaction between C-CPE and claudin-4. (A) Pull-down assay. Caco-2 lysate was incubated with C-CPE or mutant C-CPE for 30 min at 37 °C. Ni-agarose was then added, and the mixture was incubated for 3 h at 4 °C. The resin was collected by centrifugation, and the bound proteins were separated by SDS-PAGE and analyzed by Western blotting with antibodies against the indicated proteins. The results are representative of three independent experiments. (B) Competitive effect of mutant C-CPEs on C-CPE-PSIF-induced cell death in CL4/L cells. CL4/L cells were treated with C-CPE or mutant C-CPE at the indicated levels for 1 h, followed by 0.2 μg/ml C-CPE-PSIF for 36 h, after which LDH release was assessed. Values are means ± S.D. ($n = 3$), and the results are representative of three independent experiments. Significant differences from C-CPE-PSIF-treated cells ($^*p < 0.05$). (C) Cytotoxicity of mutant C-CPE-PSIF. CL4/L cells were treated with C-CPE-PSIF or mutant C-CPE-PSIF proteins for 36 h at the indicated levels, after which LDH release was measured. Values are means ± S.D. ($n = 3$). The results are representative of three independent experiments. Significant differences from C-CPE-PSIF-treated cells ($^*p < 0.05$).

amino acids and the three tyrosine residues in the 16 amino acids at position 306, 310, and 312 are involved in the C-CPE activities [10–12]. Site-directed mutagenesis of Tyr to Ala revealed that Tyr306 plays a pivotal role in interaction of C-CPE with claudin-4 and modulation of TJ-barrier by C-CPE [12]. To further examine the contribution of Tyr306 to binding of C-CPE to claudin-4 and TJ modulation by C-CPE, we generated additional mutants, namely, Y306F, Y306W, and Y306K. To evaluate effect of substitution of Tyr306 with Phe, Trp, and Lys on the interaction of C-CPE with claudin-4, we performed a pull-down assay using Caco-2 lysates. As shown in Fig. 1A, substitution of Tyr with Phe (Y306F) did not cause a noticeable effect on claudin-4 binding; however, mutation of Tyr306 to Trp or Lys decreased the binding of claudin-4 in this assay. To confirm the interaction of C-CPE mutants with claudin-4, we performed an assay based on the cytotoxicity of a fusion of C-CPE and PSIF. PSIF is a fragment of *pseudomonas* exotoxin A that inhibits protein synthesis when it enters the cell [15–17]. PSIF cannot invade into cells and does not show any cytotoxicity because of lacking the cell binding domain. So it must be fused with a ligand molecule to be imported into the cell. We previously showed that the C-CPE-PSIF fusion protein is toxic to claudin-4-expressing cells [14]. We further examined whether mutant C-CPE can reduce the toxicity of C-CPE-PSIF in claudin-4-expressing cells. As shown in Fig. 1B, pretreatment of claudin-4-expressing cells with C-CPE, Y306F or Y306W, but not Y306K, dose-dependently inhibited the toxicity of C-CPE-PSIF. Especially, the affinity of the Y306F mutant was increased compared with that of C-CPE. Y306W had the moderate affinity for claudin-4. In contrast, substitution of Lys for Y306 almost eliminated the ability of C-CPE to interact with claudin-4. Then, we examined the effect of mutant C-CPE-PSIF fusion proteins on claudin-4-expressing cells. As shown in Fig. 1C, Y306A-PSIF was less toxic than C-CPE-PSIF, indicating that Tyr306 participates in the interaction between C-CPE and claudin-4. The data on Y306A corresponded to the data of Y306A in competition assay using C-CPE-PSIF [12]. We also found that substitution of C-CPE with Y306K in C-CPE-PSIF reduced its toxicity and displacement of C-CPE with Y306F in C-CPE-PSIF partly enhanced cytotoxicity of C-CPE-PSIF corresponded to their binding of claudin-4 (Fig. 1C). The order of affinity for claudin-4 is Y306F > C-CPE > Y306W > Y306A > Y306K.

3.2. *Effects of the Y306F, Y306W, and Y306K mutants on the TJ barrier function*

We next investigated the effects of Y306F, Y306W, and Y306K on the barrier function of TJs in Caco-2 monolayer cells. As indicated in Fig. 2A, all of the mutants decreased the TER values, although their abilities varied. Substitution of Tyr306 with Lys but not with Phe or Trp reduced the ability to modulate the TJ barrier function. We further examined the dose-dependency of the effects of Y306F and Y306W on the

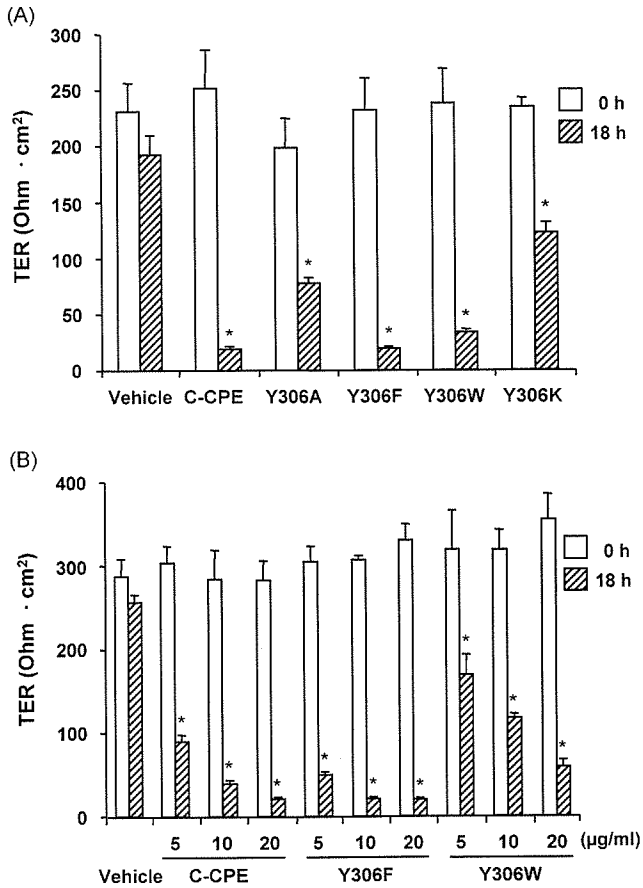

Fig. 2 – Effects of Tyr306 mutants of C-CPE on TJ barrier function. TJ-developing Caco-2 cells were grown in Transwell chambers and treated with vehicle or mutant C-CPEs from basal side of the Transwell chamber at 20 μg/ml (A) or at the indicated concentrations (B). After 18 h, TER values were determined. Values are means ± S.D. ($n = 4$), and the results are representative of three independent experiments. Significant difference from the value at 0 h ($^*p < 0.05$).

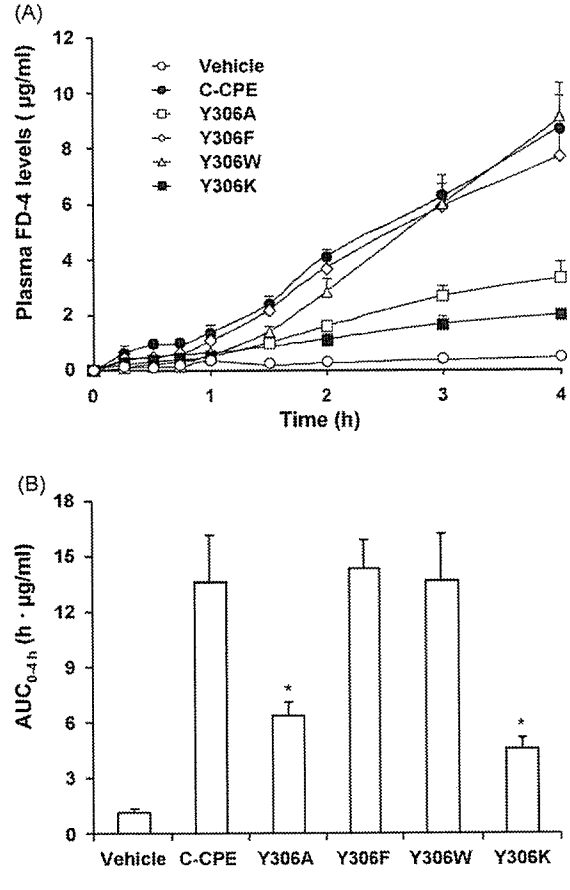

Fig. 3 – Effects of Y306 mutants on rat jejunal absorption. Rat jejunum was treated with FD-4 (10 mg/ml) with or without mutant C-CPEs (0.2 mg/ml). (A) The FD-4 levels in plasma collected from the jugular vein were determined at the indicated times. Values are means ± S.E.M. ($n = 4$), and the results are representative of three independent experiments. (B) The $AUC_{0-4\ h}$. Values are means ± S.E.M. ($n = 4$), and the results are representative of three independent experiments. Significant differences from C-CPE-treated group ($^*p < 0.05$).

TJ barrier function. As shown in Fig. 2B, at 5 and 10 μg/ml, Y306F had slightly stronger effects on the TJ barrier function than C-CPE, whereas Y306W was weaker than C-CPE. These results agree with those from our studies of claudin-4 binding (Fig. 1).

3.3. *Effects of Y306F, Y306W, and Y306K mutants on absorption in rat jejunum*

We previously reported that C-CPE enhances jejunal absorption in rats [10]. Therefore, we checked the effects of the mutations on the ability to enhance rat jejunal absorption. We used an in situ loop assay that employs FD-4 (fluorescein isothiocyanate-conjugated dextran) as a model drug absorbed via the paracellular route [18]. This assay allows investigation of the transport of a model drug from the intestine to the systemic circulation. Finally, we investigated the effects of the Y306F, Y306W, and Y306K mutants on jejunal absorption of FD-4 in rat. As shown in Fig. 3A and B, mutation of Tyr306 to Phe or Trp did not influence the absorption-enhancing activity of C-CPE, but mutation to Lys reduced the enhancing activity ($AUC_{0-4\ h}$ = 13.6 and 4.6 h μg/ml for C-CPE and Y306K, respectively). These results indicate that the aromatic ring of Tyr306 is important for the absorption-enhancing effect of C-CPE.

4. Discussion

Modulation of the barrier function of specific claudins is an attractive strategy for the development of a drug delivery system, but use of C-CPE is the only method that has been explored because it is the only molecule known to modulate the barrier function of a claudin [9]. We previously showed that modulation of claudin with C-CPE can be used to enhance drug delivery [10]. Identification of the functional domain of C-CPE should allow development of additional claudin-4 modulators. We previously found that Tyr306 is a key residue

for the disruption of the barrier function of TJs by C-CPE and for its interaction with claudin-4 [12], and we show here that aromatic and hydrophobic properties of Tyr at position 306 is partly important for the C-CPE activities.

Because CPE is a foodborne toxin in humans, its functional domains have been mapped in many studies. C-CPE is the C-terminal fragment of CPE and is responsible for binding of CPE to its receptor [19]. Hanna et al. showed that the C-terminal 30 amino acids of CPE mediates binding to its receptor, claudin-4 [20]. We previously show that deletion of the C-terminal 16 amino acids of C-CPE eliminates the TJ-modulating activity of C-CPE [11] and that the tyrosine residues (Tyr306, 310, 312) in the 16 amino acids of C-CPE are responsible for the C-CPE activity [12]. Substitution of Tyr306 with Ala reveals that Tyr306 is a pivotal residue for the abilities of C-CPE to bind claudin-4 and modulate the TJ barrier [12].

What means the attenuation of the C-CPE activities by mutation of Tyr306 to Ala in C-CPE? Tyr is a polar, hydrophobic and aromatic residue, but Ala is a non-polar, non-hydrophobic and non-aromatic residue. Therefore, we suspected that polar, hydrophobic and/or the aromatic property of Tyr is important for the activities of C-CPE. We prepared Y306F (aromatic and hydrophobic mutant), Y306W (aromatic, hydrophobic and polar mutant), and Y306K (polar and positive charged mutant) mutants and examined their abilities to modulate the TJ barrier function and bind claudin-4. Replacement of Tyr with Phe did not affect these activities, whereas replacement with Lys attenuated these activities, and mutation to Trp caused a partial reduction in both activities. These findings suggest that the aromatic and hydrophobic properties of Tyr at position 306 are important for the activities of C-CPE and hydrogen bonding potential of Tyr at position 306 is not essential for them.

How does Tyr306 contribute to modulation of the TJ barrier function and to the interaction with claudin-4? Considering the mode of action of C-CPE as a claudin modulator, this leaves the question of whether it is possible to separate the ability of C-CPE to modulate the TJ barrier function and its ability to interact with claudin. Deletion of the C-terminal region of C-CPE and substitution of Tyr306 with Ala in C-CPE eliminate these activities [10–12]. Similarly, we showed here that mutation of Tyr306 to Lys reduces both the ability to modulate the TJ barrier function and the ability to bind claudin-4. These findings suggest that the abilities of C-CPE to bind to claudin-4 and modulate TJ-barrier cannot be separated in Tyr306 mutants. Tsukita lab found that CPE interacted with claudin via extracellular loop domain of claudin and claudin was degraded by endocytotic pathway in C-CPE-treated cells [9,21]. We also found that loss of interaction of C-CPE with claudin-4 by deletion of the C-terminal C-CPE [10,11]. These findings indicate that interaction of C-CPE with claudin is the first step in the modulation of TJ-barrier by C-CPE. Tyr306 may be partly critical for interaction of C-CPE with claudin. Since Y306F mutant binds to claudin-4, interaction of C-CPE with claudin-4 may not be mediated by hydrogen bond. Mutation of Tyr to Trp at position 306 resulted in C-CPE with subtle differences in claudin-4 binding. In contrast, Y306K and Y306A mutants reduced binding to claudin-4. Taken together, the hydrophobicity at position 306 may be important for binding of C-CPE to claudin-4. An aromatic/polar amino acid at position 306 might be critical for the correct folding of C-CPE, which might expose other residues and allow them to reach their target site on claudin-4.

Determination of the three-dimensional structures of CPE and claudin is critical for elucidation of the precise mechanism of interaction between C-CPE and claudin-4, but, because these proteins are hydrophobic, this has not yet been accomplished. In the meantime, our findings should help to clarify how these two proteins interact and to prepare a novel claudin-modulator using C-CPE as a prototype.

Acknowledgements

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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.

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).

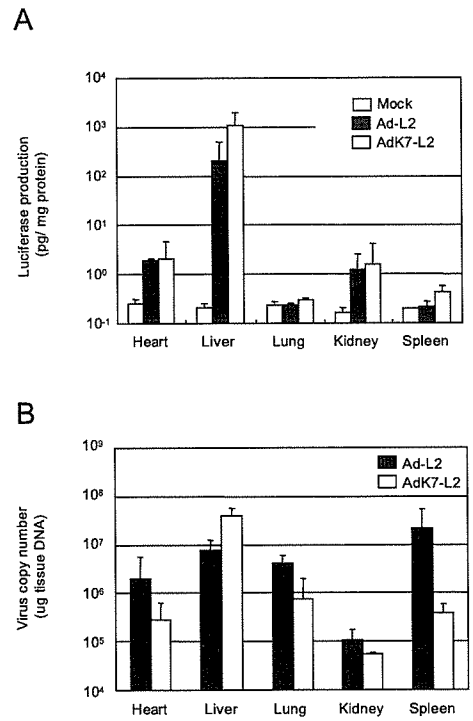

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production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