Ad/ΔF(FG)ΔP-S35-L2 or Ad-L2 (Fig. 2). SK HEP-1 cells express both CAR and α_v integrin (Koizumi *et al.*, 2001, 2003b). To measure the internalization of Ad particles into the cells, Ad genome DNA in the cells after 1.5 hr of transduction with each Ad vector was also quantified with the TaqMan fluorogenic detection system. Viral particles associated with the cellular surface were removed by trypsin–EDTA–PBS and DNase I–$MgCl_2$–PBS treatment as described in Materials and Methods. Cells transduced with Ad/ΔF(AB)ΔP-S35-L2 showed much lower luciferase production than those transduced with Ad/ΔF(FG)ΔP-S35-L2. Ad/ΔF(AB)ΔP-S35-L2 mediated only approximately 0.003% of the luciferase production of Ad-L2, whereas Ad/ΔF(FG)ΔP-S35-L2 mediated approximately 0.42% of that of Ad-L2 (Fig. 2A). In contrast, the amounts of Ad/ΔF(AB)ΔP-S35-L2 DNA and Ad/ΔF(FG)ΔP-S35-L2 DNA in SK HEP-1 cells were only 10-fold lower than those of Ad-L2 DNA. The amounts of Ad/ΔF(AB)ΔP-S35-L2 DNA in the cells were similar to those of Ad/ΔF(FG)ΔP-S35-L2 DNA (Fig. 2B).

Because Ad/ΔF(AB)ΔP-S35-L2 showed extremely low transduction activity, we examined luciferase production in SK HEP-1 cells transduced with Ad/ΔF(AB)ΔP-S35-L2 in the presence of SuperFect (polyamidoamine dendrimer reagent; Qiagen). Ad/ΔF(AB)ΔP-S35-L2 mediated high levels of luciferase production in a dose-dependent manner with SuperFect (Fig. 2C). Therefore, low luciferase production by Ad/ΔF(AB)ΔP-S35-L2 is likely due to a lack of specific binding activity between the virus and target cells and to endosomal escape, but it was not due to the virus being defective. These results suggest that the abolishment of CAR, integrin, and HSG binding of Ad vectors significantly reduces transduction efficiency and that the four-amino acid mutation of the AB loop of the fiber knob reduces transduction to a greater extent than does the four-amino acid deletion of the FG loop of the fiber knob.

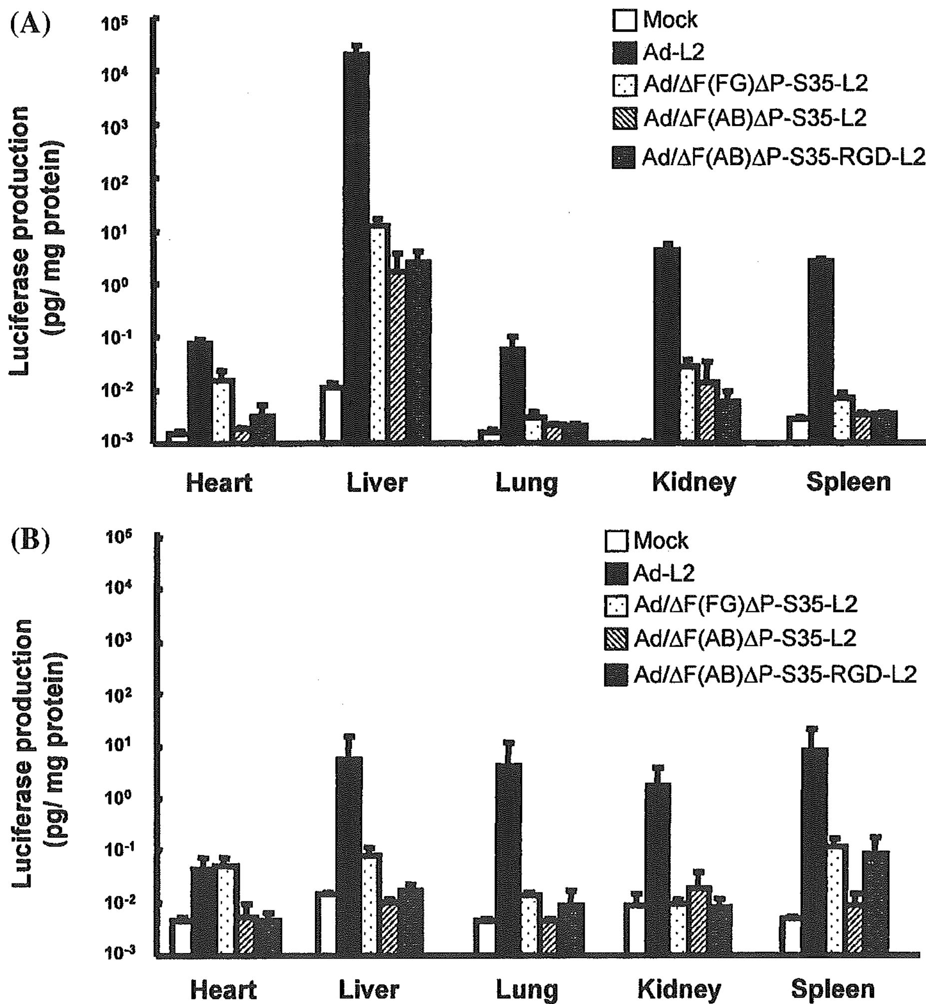

FIG. 3. Luciferase production in mice after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected into mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested and luciferase production was measured by a luciferase assay system. All data represent the means ± SD of four to six mice.

Gene transfer in vivo

Next, to examine whether natural Ad tropism to tissues, including liver, can be more suppressed by Ad/ΔF(AB)ΔP-S35-L2 in comparison with Ad/ΔF(FG)ΔP-S35-L2, each Ad vector was administered to mice by either intravenous (3.0×10^{10} VP) or intraperitoneal (1.0×10^{11} VP) injection, and luciferase production in the organ was measured (Figs. 3 and 4). In the case of intraperitoneal injection, a high dose of Ad vector (1.0×10^{11} VP) was injected because luciferase production was not detected in mouse tissue after intraperitoneal injection of 3.0×10^{10} VP of either Ad/ΔF(FG)ΔP-S35-L2 or Ad/ΔF(AB)ΔP-S35-L2. With intravenous injection, Ad/ΔF(AB)ΔP-S35-L2 mediated approximately 15,000-fold lower liver transduction than Ad-L2, and resulted in approximately 10-fold lower liver transduction compared with Ad/ΔF(FG)ΔP-S35-L2 (Fig. 3A). A similar pattern was observed in the heart, lung, kidney, and spleen, although the absolute levels of luciferase production were much lower compared with those in the liver.

With intraperitoneal injection, Ad-L2 mediated similar levels of luciferase production in the liver, lung, kidney, and spleen (Fig. 3B). The suppressive pattern of luciferase production in each organ after intraperitoneal injection of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 was similar to that after intravenous injection. Ad/ΔF(AB)ΔP-S35-L2 showed much more reduced luciferase production in the organs than did Ad/ΔF(FG)ΔP-S35-L2 (Fig. 3B). Luciferase production in each organ after intraperitoneal injection of Ad/ΔF(AB)ΔP-S35-L2 was at almost background levels. These results indicate that the triple-mutant Ad vector containing a mutation of the AB loop of the fiber knob exhibits much lower luciferase production than does the triple-mutant Ad vector containing a mutation of the FG loop of the fiber knob, in both intravenously and intraperitoneally injected mice.

Distribution of Ad vectors after systemic administration

To examine the biodistribution of Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad-L2 in mice at a early stage after intravenous (3×10^{10} VP) and intraperitoneal (1×10^{11} VP) injection, the amounts of Ad DNA in organs 3 hr after Ad vector injection were measured with the TaqMan fluorogenic detection system. The amounts of Ad DNA in organs after intravenous injection showed no significant difference among mice injected with Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad-L2 (Fig. 4A), although the amounts of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 in the kidney were less than that of Ad-L2. In the case of intraperitoneal injection, Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 showed higher or similar amounts of Ad DNA in the liver or spleen, respectively, than Ad-L2 (Fig. 4B).

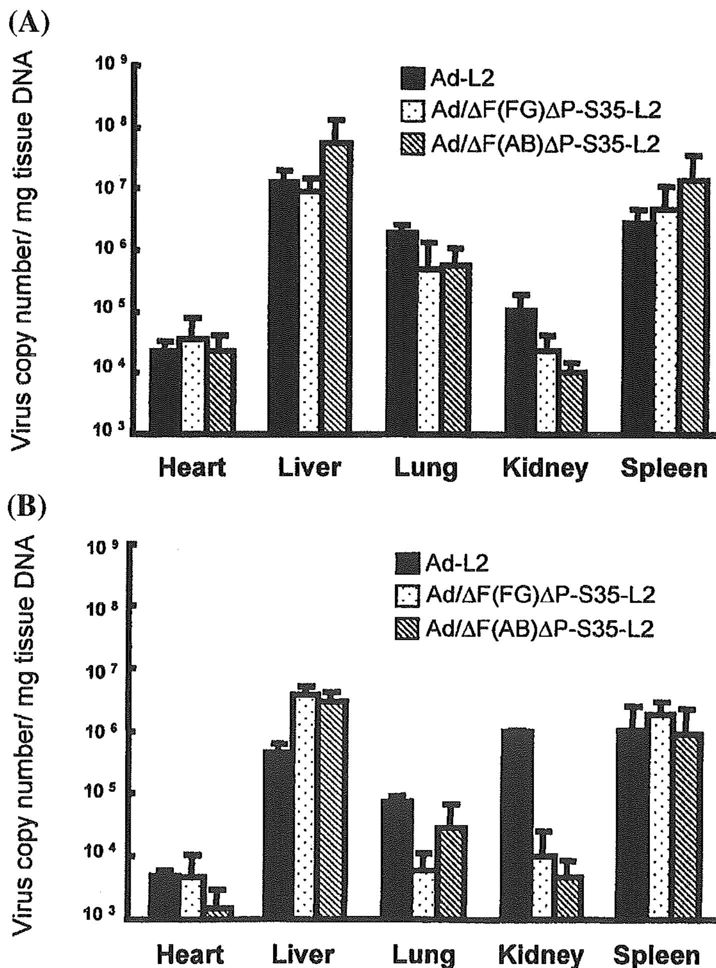

FIG. 4. Biodistribution of viral DNA after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected into mice. Three hours later, the heart, lung, liver, kidney, and spleen were harvested and Ad vector DNA was measured with the quantitative TaqMan PCR assay. All data represent the means ± SD of four to six mice.

Less Ad/ΔF(AB)ΔP-S35-L2 accumulated in the heart, lung, and kidney compared with Ad-L2. The data regarding luciferase production (Fig. 3) and the amounts of Ad DNA in most organs, especially the liver (Fig. 4), showed discrepancies in the cases of both intravenous and intraperitoneal injection.

Amounts of Ad vector DNA in liver parenchymal and nonparenchymal cells

Next, to examine why there is an especially large difference between luciferase production and Ad DNA accumulation in the liver, the amounts of Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad-L2 delivered to parenchymal cells (PCs; hepatocyte) and nonparenchymal cells (NPCs; Kupffer cells and endothelial cells) 3 hr after injection were measured with the TaqMan fluorogenic detection system (Fig. 5). In the case of intravenous injection of Ad vector at 3×10^{10} VP, more Ad-L2 DNA was found in PCs than in NPCs, whereas there was less Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 DNA in PCs than in NPCs (Fig. 5A). This finding is consistent with our previous reports based on analysis by semiquantitative PCR (Koizumi *et al.*, 2003a). In the case of intraperitoneal injection of Ad vector at 1×10^{11} VP, Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad-L2 DNA accumulated more in NPCs than in PCs (Fig. 5B). Thus, lower luciferase production in the liver after intravenous and intraperitoneal injection of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 would be partly due to higher accumulation of vectors in NPCs. The NPCs might take up Ad via phagocytosis and resolve viral DNA, resulting in lower gene expression.

Blood clearance of Ad vectors

To examine the biodistribution in more detail, the blood clearance rates of Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad-L2 in mice were measured with the TaqMan fluorogenic detection system (Fig. 6). In the case of intravenous injection, blood clearance curves for Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad-L2 were similar, and all the vectors showed rapid decrease from the bloodstream (Fig. 6A). In the case of intraperitoneal injection, Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 showed similar blood clearance curves. The amounts of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 DNA were approximately 10-fold higher than those of Ad-L2 DNA between 60 and 120 min after injection (Fig. 6B). The area under the curve (AUC_{2-180}) values of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 were 5- to 7-fold higher than that of Ad-L2 (data not shown). Higher levels of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 were found to be introduced into the bloodstream from the intraperitoneum than Ad-L2.

Liver serum enzymes and serum interleukin-6 levels after administration of Ad vector

Systemic administration of Ad vectors results in the initiation of inflammation and strong innate immunity responses in animals and humans (Schnell *et al.*, 2001; Muruve, 2004), and this toxicity limits the utility of Ad vectors for gene therapy. To evaluate the toxicity of each Ad vector, we measured the levels of AST, ALT, and IL-6 in serum after systemic administration. After injection of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 in mice (both by intravenous and intraperitoneal injection), the levels of AST and ALT in serum were similar to those in nontreated mice, suggesting that Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 did not show liver toxicity (Fig. 7). In contrast, Ad-L2 led to high levels of AST and ALT in serum after intravenous injection (Fig. 7A). In the case of IL-6, neither intravenous nor intraperitoneal injection of Ad/ΔF(AB)ΔP-S35-L2 or Ad/ΔF(FG)ΔP-S35-L2 mediated IL-6 production, whereas injection of Ad-L2 led to high levels of IL-6 in serum (Fig. 8). These results suggest that Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 show less liver toxicity and innate immunity reaction (IL-6 production) after systemic administration.

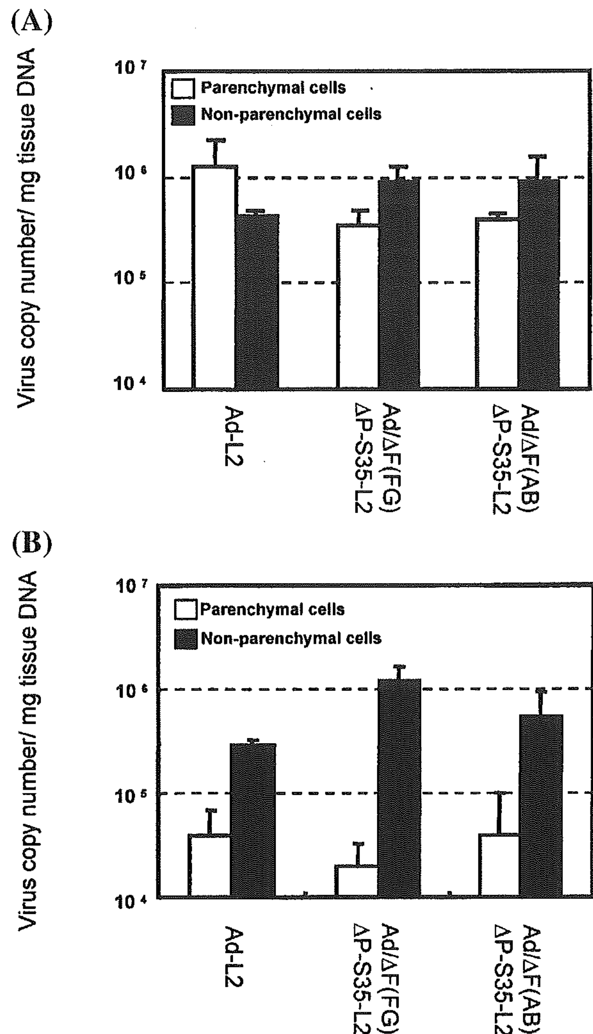

FIG. 5. Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected into mice. Collagenase perfusion was performed 3 hr after injection of Ad vector to separate liver PCs and NPCs. Total DNA, including Ad vector DNA, was isolated from the cells, and Ad vector DNA was measured by quantitative TaqMan PCR assay. All data represent the means ± SD of four to six mice.

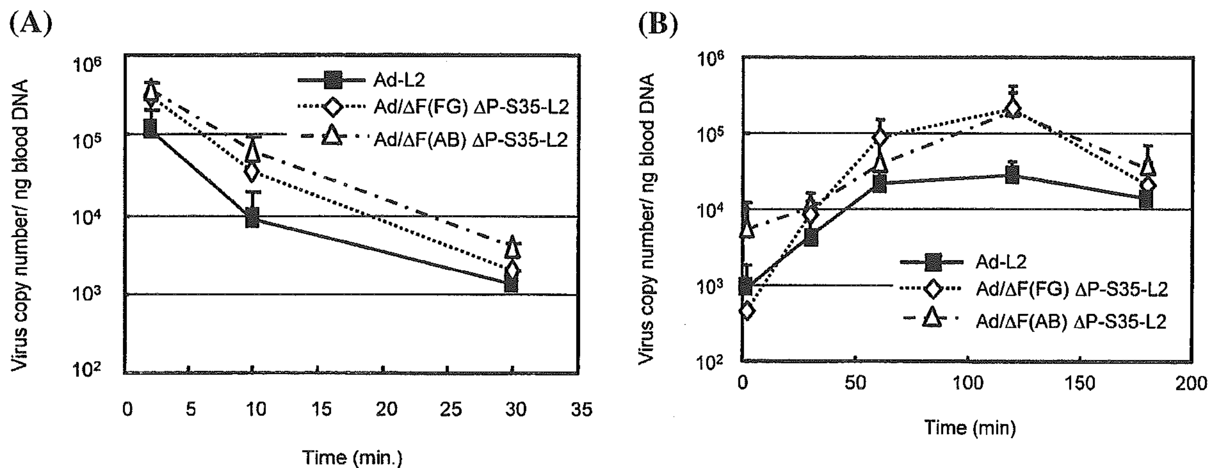

FIG. 6. Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected, and blood was drawn by retroorbital bleeding at the indicated times postinjection. Total DNA, including Ad vector DNA, was isolated from the blood, and Ad vector DNA was measured by quantitative TaqMan PCR assay. All data represent the means ± SD of four to six mice.

Inclusion of RGD ligand into the fiber knob in triple-mutant Ad vectors

For the development of a targeted Ad vector, addition of foreign ligands into a viral capsid that no longer infects cells is required. For this purpose, Ad/ΔF(AB)ΔP-S35-RGD-L2, in which the RGD peptide was introduced into the HI loop of the fiber knob of Ad/ΔF(AB)ΔP-S35-L2, was constructed, and gene transfer activity was measured in SK HEP-1 cells (Fig. 9A). Ad/ΔF(AB)ΔP-S35-RGD-L2 showed 100-fold higher luciferase production in SK HEP-1 cells than did Ad/ΔF(AB)ΔP-S35-L2 (Fig. 9A). In the inhibition experiment using RGD peptide, luciferase production in cells transduced with Ad/ΔF(AB)ΔP-S35-RGD-L2 was suppressed by RGD peptide in a dose-dependent fashion, suggesting that Ad/ΔF(AB)ΔP-S35-RGD-L2 mediates gene transfer through RGD peptides in the fiber knob (Fig. 9B).

Next, to examine whether Ad/ΔF(AB)ΔP-S35-RGD-L2 mediates luciferase production *in vivo* in a manner different from Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-RGD-L2 was administered to mice by either intravenous (3.0×10^{10} VP) or intraperitoneal (1.0×10^{11} VP) injection, and luciferase production in organs was measured (Fig. 3). Data suggest that addition of RGD peptide to the triple-mutant Ad vector does not change the biodistribution *in vivo*, although intraperitoneal injection of Ad/ΔF(AB)ΔP-S35-RGD-L2 mediated slightly higher luciferase production in the spleen compared with Ad/ΔF(AB)ΔP-S35-L2.

DISCUSSION

In this study, we generated a new Ad vector with a four-amino acid mutation of the AB loop in the fiber knob (T489, A490, Y491, and T492), deletion of the RGD motif of the penton base, and substitution of the fiber shaft domain for that derived from Ad type 35, and demonstrated that this triple-mutant Ad vector shows significantly lower gene transfer activity (both *in vitro* and *in vivo*). The triple-mutant Ad vector containing a mutation of the AB loop in the fiber knob mediated much lower gene transfer activity than the previously generated triple-mutant Ad vector containing a mutation of the FG loop in the fiber knob (Koizumi *et al.*, 2003a). Furthermore, the triple-mutant Ad vector was less toxic, and showed almost background levels of both liver serum enzymes (AST and ALT) and IL-6 in mouse serum.

Ad vectors show nonspecific tissue distribution after *in vivo* gene transfer. This distribution is due largely to the relatively broad expression of CAR, α_v integrin, and HSGs; the size of sinusoidal fenestrae (Fechner *et al.*, 1999; Lievens *et al.*, 2004); and the complement system (Zinn *et al.*, 2004). To generate targeted Ad vectors, several groups have reported CAR binding-ablated Ad vectors with an AB or FG loop mutation of the fiber knob (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Asaoka *et al.*, 2000; Alemany and Curiel, 2001; Einfeld *et al.*, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002; Smith *et al.*, 2002). However, there has been no report on the difference in gene transfer activity (*in vitro* and *in vivo*) between Ad vectors with an AB loop mutation and those with an FG loop mutation. The present study shows that mutation of the AB loop in the fiber knob is better than deletion of the FG loop for lowering transgene expression, at least with the triple-mutant Ad vector. Cells transduced with Ad/ΔF(AB)ΔP-S35-L2 or Ad/ΔF(FG)ΔP-S35-L2 produced luciferase at rates of only 0.003 and 0.42%, respectively, relative to the rate of luciferase production in cells transduced with Ad-L2 (Fig. 2A). The FG loop mutation in the fiber knob might continue to facilitate a weak interaction between CAR and the fiber knob. One of the interesting findings is that the amounts of Ad/ΔF(AB)ΔP-S35-L2 DNA and Ad/ΔF(FG)ΔP-S35-L2 DNA in the cells were only 10-fold lower than those of Ad-L2 DNA, even after the cells were treated with trypsin–EDTA and DNase I (Fig. 2B). Therefore, the cells would take up considerable amounts of Ad/ΔF(AB)ΔP-S35-L2 and Ad/ΔF(FG)ΔP-S35-L2 nonspecifically, although neither vector mediated luciferase production.

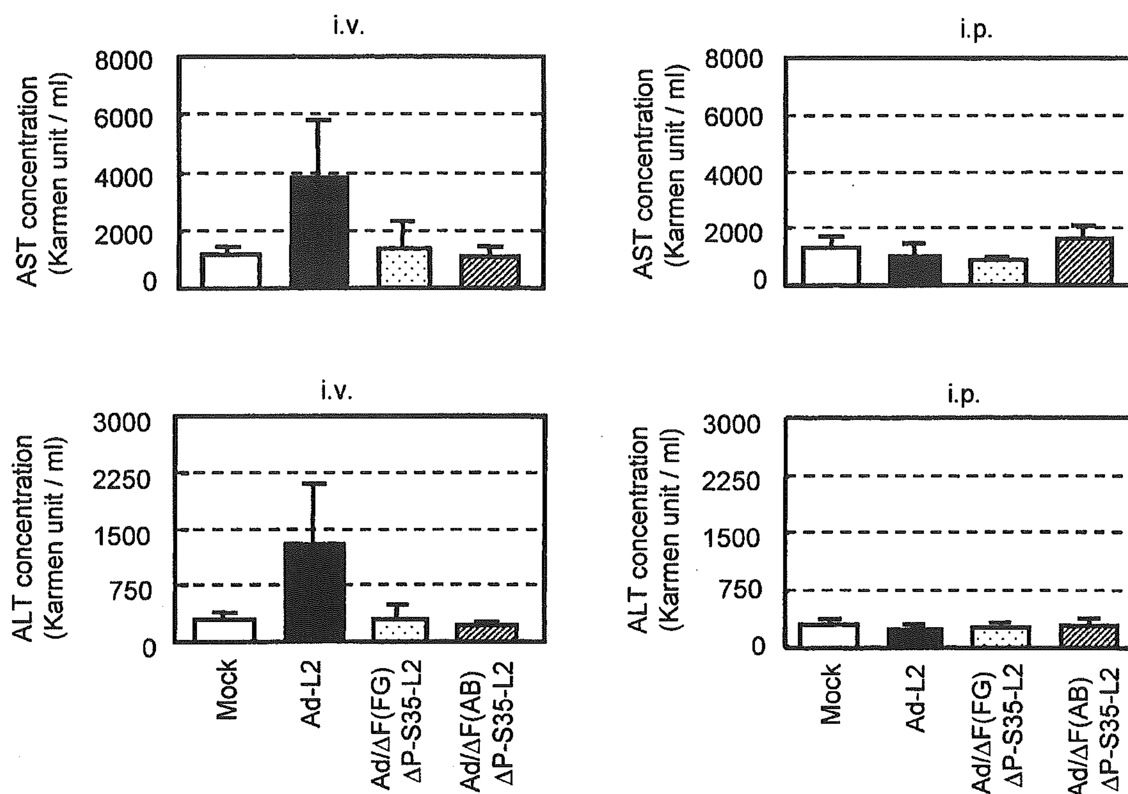

FIG. 7. Serum enzymes levels after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Blood samples were collected from the inferior vena cava 48 hr after intravenous (3.0×10^{11} VP) or intraperitoneal (1.0×10^{11} VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. Serum samples were collected into separate tubes containing no anticoagulant for coagulation, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in serum were measured with a Transaminase-CII kit. All data represent the means ± SD of four mice.

We demonstrated that the newer triple-mutant Ad vector containing a mutation of the AB loop mediates approximately 15,000- and 500-fold lower mouse liver transduction by intravenous and intraperitoneal injection, respectively, than the conventional Ad vector (Fig. 3). However, the amounts of triple-mutant Ad vector DNA in the liver after intravenous or intraperitoneal injection were similar to or higher than those with the conventional Ad vector (Fig. 4). The difference between luciferase production and Ad DNA accumulation in the liver would be due to higher accumulation of triple-mutant Ad vector DNA in the NPCs (Kupffer cells and endothelial cells) (Fig. 5) as well as to nonspecific viral uptake in the liver. Because higher amounts of the triple-mutant Ad vector were taken up nonspecifically into the cultured cells (Fig. 2B), the liver cells *in vivo* would also take up large amounts of virus nonspecifically. Our previous report showed that most Ad DNA (especially the triple-mutant Ad DNA) taken up in NPCs disappears 48 hr after intravenous administration (Koizumi *et al.*, 2003a). Triple-mutant Ad vectors in NPCs might be resolved, resulting in significantly lower gene expression in the liver. Furthermore, Miyazawa *et al.* have reported that exchanging the Ad type 5 fiber (subgroup C) for the Ad type 7 fiber (subgroup B) on an Ad type 5 capsid resulted in altered cellular trafficking compared with parental Ad type 5 (Miyazawa *et al.*, 1999, 2001). Therefore, even if the triple-mutant Ad vector, in which the Ad type 5 fiber shaft was exchanged for the Ad type 35 fiber shaft (subgroup B), was taken up into cells, it might have defects in viral escape from the endosome to the cytoplasm (Nicklin *et al.*, 2005).

We and others have reported that the conventional Ad vector has a half-life in the bloodstream of approximately 2 min after intravenous injection (Alemany *et al.*, 2000; Alemany and Curiel, 2001; Koizumi *et al.*, 2003a; Sakurai *et al.*, 2003). The triple-mutant Ad vector and the conventional Ad vector presented similar clearance kinetics from the circulation after intravenous injection (Fig. 6A). In the case of intraperitoneal injection, the AUC_{2-180} value of the triple-mutant Ad vector in the bloodstream was approximately five to seven times higher than that of the conventional Ad vector (Fig. 6B). It remains unclear why intraperitoneally injected vectors persist longer in the blood (Akiyama *et al.*, 2004). The vector might associate with blood factors or cells (Shayakhmetov *et al.*, 2005). It was also found that intraperitoneally injected vectors accumulated more in NPCs than in PCs (Fig. 5B). This NPC-mediated uptake might be an obstacle for the targeted Ad vector when it is intraperitoneally injected. Because the present vector has no targeted ligands, more detailed studies should be done after high-affinity ligands are displayed on the vectors. If high levels of NPC-mediated uptake were avoided by the addition of ligands,

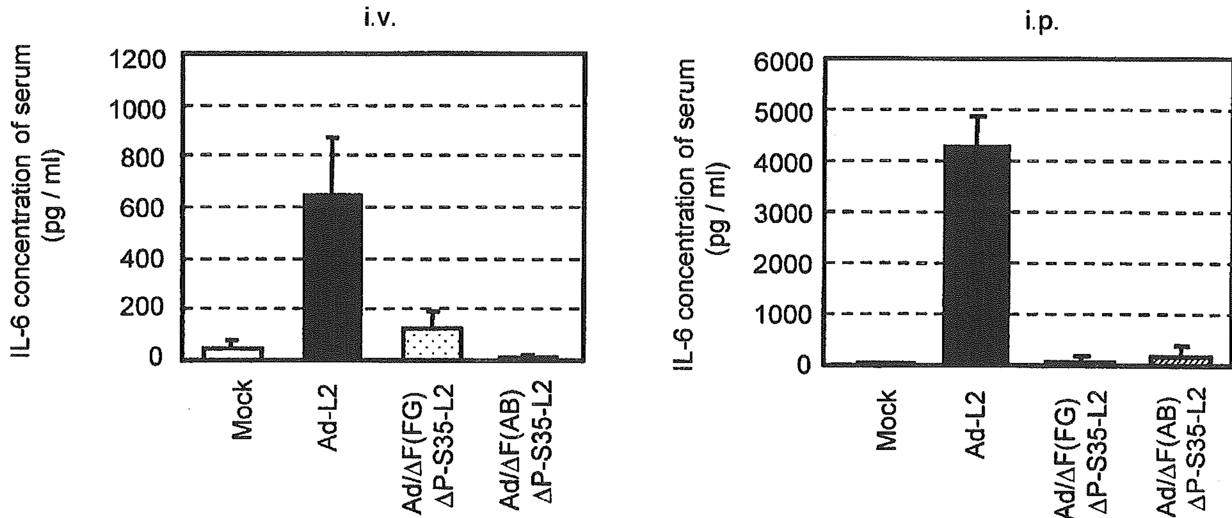

FIG. 8. Interleukin (IL)-6 levels in serum after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Blood samples were collected from the inferior vena cava 3 hr after intravenous (3.0×10^{11} VP) or intraperitoneal (1.0×10^{11} VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. Serum samples were collected into separate tubes containing no anticoagulant for coagulation, and IL-6 levels in the serum were measured by ELISA. All data represent the means ± SD of six mice.

the increased persistence of the vector in the blood in the case of intraperitoneal injection might give us a way to overcome obstacles to the development of targeted Ad vectors.

In the *in vivo* viral uptake experiment, the yield of viral DNA from total liver (Fig. 4) was an order of magnitude more than the total yield obtained from PCs and NPCs (Fig. 5). We speculated that extracellular virus, which would be present in the yield obtained from total liver but not in the yield obtained from fractionated cells, might be involved, because extracellular virus would be moved by collagenase treatment into the fractionated cells. To demonstrate this, we examined the effect of collagenase or trypsin treatment on the amounts of viral DNA in cultured cells. SK HEP-1 cells were transduced with Ad-L2 or Ad/ΔF(AB)ΔP-S35-L2 (3000 VP/cell). After a 3-hr culture period, the cells were washed with PBS, collagenase (0.01%), or trypsin (0.025%). The amounts of Ad genomic DNA in cells were

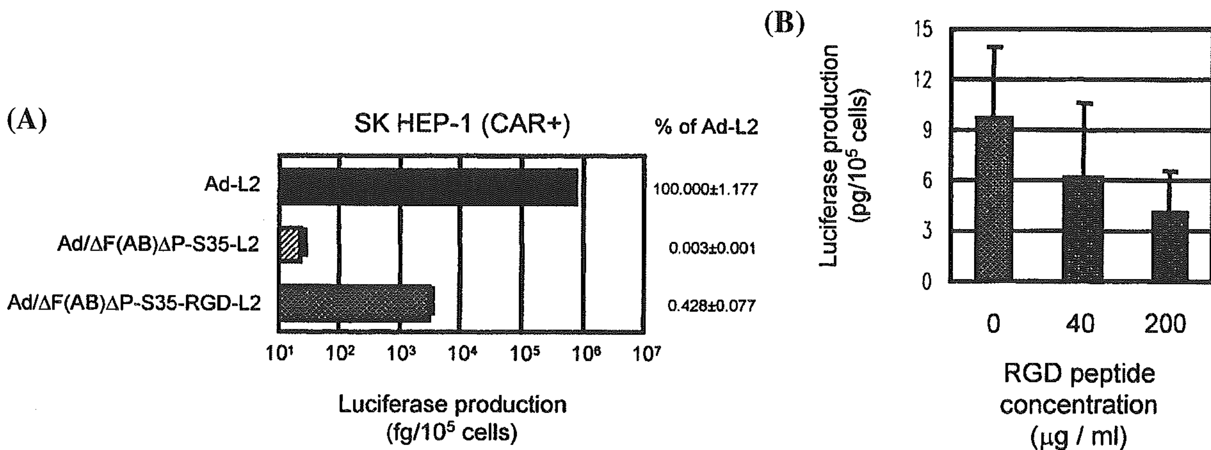

FIG. 9. Luciferase production in human cells transduced with Ad vectors containing RGD motif in the fiber knob. (**A**) Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2. SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 for 1.5 hr. After culture for 48 hr, luciferase production in the cells was measured by a luciferase assay system. The data are expressed as means ± SD ($n = 4$). The relative expression levels are described by designating the value of Ad-L2 as 100. (**B**) Effects of RGD peptide on the transduction efficiency of Ad/ΔF(AB)ΔP-S35-RGD-L2 into SK HEP-1 cells. SK HEP-1 cells were preincubated with RGD peptide (0, 1.6, 8, or 40 μg/ml) for 10 min. The cells were then transduced with 300 VP/cell of Ad/ΔF(AB)ΔP-S35-RGD-L2 for 0.5 hr in the presence of RGD peptide. After culture for 48 hr, luciferase production was measured by a luciferase assay system. The data are expressed as means ± SD ($n = 6$).

quantified with the TaqMan fluorogenic detection system. Data showed that collagenase or trypsin treatment decreased 2- to 3.5-fold the amounts of Ad DNA in cells (data not shown), suggesting that nonspecific viral association would lead to the overestimation of viral uptake by the cells. Therefore, the difference in the yields between Figs. 4 and 5 would be reasonable.

The initiation of inflammation and strong innate immunity responses occur after systemic administration of Ad vectors to animals and humans, and this toxicity limits the utility of Ad vectors for gene therapy (Muruve, 2004). Increased cytokine production after injection of Ad vectors was reported to be due to the introduction of input Ad vectors to Kupffer cells in the liver and dendritic cells (Lieber *et al.*, 1997; Schnell *et al.*, 2001; Morral *et al.*, 2002; Reid *et al.*, 2002; Philpott *et al.*, 2004). Lieber *et al.* have reported that IL-6 production in mice after injection of Ad vectors was decreased by preinjection of $GaCl_2$, which can decrease the levels of Kupffer cells in mouse liver (Lieber *et al.*, 1997). On the other hand, Muruve reported that Kupffer cells avidly take up systemically administered Ad vectors, but the blockade of Kupffer cells has minimal impact on the innate immune response in the liver (Muruve, 2004). Although our experiment showed that large amounts of the triple-mutant Ad vector accumulated in the NPC fraction, which contains Kupffer cells and liver sinusoidal (endothelial) cells, IL-6 was not produced in mice after injection of the triple-mutant Ad vector (Fig. 8). Therefore, Ad vectors would be capable of inducing IL-6 production in cells other than Kupffer cells. De Geest *et al.* reported that the spleen, not the liver, is the major site of IL-6 production after Ad vector transfer (De Geest *et al.* 2005), although in the present study the triple-mutant Ad vector accumulated in the spleen as much as did the conventional Ad vector (Fig. 4). There are several possible reasons why the triple-mutant Ad vector does not mediate IL-6 production *in vivo*. Philpott *et al.* have reported that maturation of dendritic cells, which are IL-6-producing cells, by infection with Ad vectors requires the RGD motif of the Ad penton base (Philpott *et al.*, 2004). The triple-mutant Ad vector without the RGD motif in the penton base would interact differently with IL-6-producing cells than would the conventional Ad vector. Liu *et al.* have reported that conventional Ad vectors are delivered into liver sinusoid cells as well as Kupffer cells after systemic injection (Liu *et al.*, 2003). Schiedner *et al.* have reported that Ad vectors activate liver endothelial cells after infection of Kupffer cells (Schiedner *et al.*, 2003). The difference in distribution between the triple-mutant Ad vector and the conventional Ad vector in liver sinusoid and Kupffer cells may contribute to IL-6 production. Furthermore, Zsengeller *et al.* demonstrated that Ad vector internalization and endosomal escape were required for cytokine induction in alveolar macrophages (Zsengeller *et al.*, 2000). The triple-mutant Ad vector might have reduced the level of endosomal escape in comparison with the conventional Ad vector. Specific viral component(s) of the Ad vector, viral distribution in the specific cell types, and/or viral distribution in the cellular compartment might determine IL-6 production. Elucidation of a mechanism for innate immune responses after administration of Ad vectors might be obtained by investigating the precise distribution of the triple-mutant Ad vector after systemic administration.

Finally, regarding the feasibility of using triple-mutant Ad vectors as targeted vectors, we constructed triple-mutant Ad vectors containing the RGD motif, which has high affinity for α_v integrins, in the HI loop of the fiber knob. This triple-mutant Ad vector with the RGD motif was found to show efficient *in vitro* gene transfer through RGD peptides in the fiber knob (Fig. 9). We also examined *in vivo* luciferase production and serum levels of AST, ALT, and IL-6 in mice after administration of this RGD motif-containing vector. However, the patterns of luciferase production *in vivo* (Fig. 3) and the serum levels of AST, ALT, and IL-6 (data not shown) postadministration were similar to those produced with the triple-mutant Ad vector without any ligands. Because the RGD peptide used in the present study was first isolated from a phage display library and used to "home" to endothelial cells in tumor tissue (Koivunen *et al.*, 1995; Pasqualini *et al.*, 1997), and because the endothelial cells in normal tissue do not express higher levels of α_v integrin than are found in tumor tissue, the RGD motif may not be the optimal peptide for increasing *in vivo* transduction efficiency after systemic injection. Another possible reason why this RGD motif-containing vector did not increase transduction *in vivo* is that the affinity of the introduced RGD peptides for integrin might be weak compared with the knob–CAR interaction. Furthermore, fiber mutation might affect encapsidation, stability, and flexibility of the vector. The resultant subtle alteration in fiber biology might negatively affect the transduction efficiency of this vector. Altered fiber biology might also be involved in the lower gene transduction efficiency of the triple-mutant Ad vector.

For the development of targeted Ad vectors, incorporation of a foreign ligand (i.e., peptide), one with high affinity for a specific cellular receptor, into the capsids of Ad vectors will also be required. The triple-mutant Ad vector was designed to have unique restriction sites (*Csp*45I or *Cla*I) in both the HI loop and the C-terminal coding region of the fiber knob (Mizuguchi *et al.*, 2001; Koizumi *et al.*, 2001, 2003b). Therefore, any targeting ligand can be easily displayed in the fiber knob of the triple-mutant Ad vector by cloning its gene into either of these regions, using simple *in vitro* ligation.

In summary, we have further improved the triple-mutant Ad vector by ablating CAR, α_v integrin, and HSG binding by introducing a mutation of the AB loop into the fiber knob (R412S, A415G, E416G, and K417G). This vector was found to mediate significantly lower tissue transduction both *in vitro* and *in vivo* (intravenous and intraperitoneal injection). Furthermore, we showed that this triple-mutant Ad vector reduces (or blunts) liver toxicity and innate immunity responses (IL-6 production). Inclusion of the RGD peptide in the HI loop of the fiber knob of the triple-mutant Ad vector restored gene transfer activity. Thus, the newer triple-mutant Ad vector will likely be a fundamental vector for targeted gene delivery.

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Approaches to improving the kinetics of adenovirus-delivered genes and gene products

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Abstract

Adenovirus (Ad) vectors have been expected to play a great role in gene therapy because of their extremely high transduction efficiency and wide tropism. However, due to the intrinsic deficiency of their immunogenic toxicities, Ad vectors are rapidly cleared from the host, transgene expression is transient, and readministration of the same serotype Ad vectors is problematic. As a result, Ad vectors are continually undergoing refinement to realize their potential for gene therapy application. Even after 1999, when a patient fatally succumbed to the toxicity associated with Ad vector administration at a University of Pennsylvania (U.S.) experimental clinic, enthusiasm of gene therapists for Ad vectors has not waned. With great efforts from various research groups, significant advances have been achieved through comprehensive approaches to improving the kinetics of Ad vector-delivered genes and gene products.
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Keywords: Immunogenic toxicities; Biodistribution; Cationic liposome; PEGylation; Helper-dependent; Targeting; In-cis acting element; Integration; Regulatable expression

Contents

1. Introduction 782
2. Immunogenic toxicities of Ad vectors 784
3. Kinetics of Ad vector-delivered gene and gene product 784
4. Approaches to improving the kinetics of Ad-delivered genes and gene products 786

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4.1. Viral genome deletion . . . 786
4.2. Modulation of the viral tropism . . . 787
4.2.1. Increasing transduction of target cells by bispecific conjugates . . . 788
4.2.2. Increasing transduction of target cells by genetic modification of the fiber . . . 788
4.2.3. Ablation of the native tropism . . . 789
4.3. Cationic liposome complexation or PEGylation . . . 791
4.4. Immune intervention . . . 791
4.5. Optimization of in-cis acting elements . . . 792
4.6. Integrating Ad vectors . . . 793
4.7. Serotype switch or animal Ad vectors for readministration . . . 794
5. Summary . . . 795
Acknowledgements . . . 795
References . . . 795

1. Introduction

Viral vectors show great promise for gene delivery in both basic research and therapeutic applications. It is vital to select the most appropriate viral vector for each specific application, and a number of factors must be taken into consideration when making such a selection. These include the efficiency and specificity with which the vector infects the target cells, the transgene size, the level and duration of the transgene expression, the question of whether regulation of the transgene is needed, and the level of toxicity that can be tolerated. There are now more than 10 viral vector types in use, derived from common human or mammalian viral pathogens including retrovirus, adenovirus, adeno-associated virus, lentivirus, herpes simplex virus, and poxvirus. However, there is no single viral vector type meeting all the requirements, and the methods for using viral vectors to deliver genes are continually being refined.

Adenoviruses (Ad) are nonenveloped viruses containing an icosahedral protein capsid with a diameter of approximately 80 nm. There are at least 51 serotypes of human Ad identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) along with Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, penton base, and fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Fig. 1). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: tail, shaft, and knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber

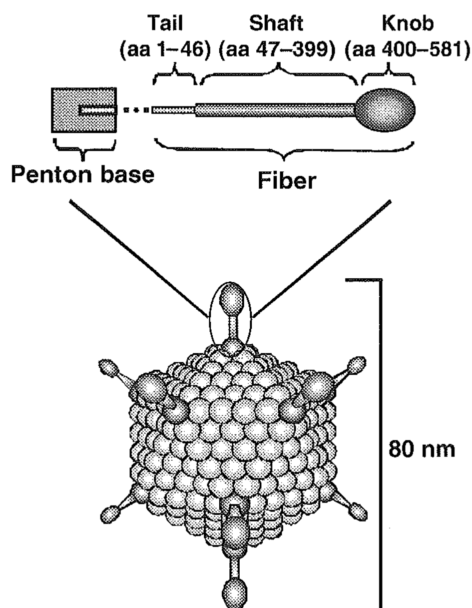

Fig. 1. Schematic diagram of a human Ad serotype 5 virion. The double stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices.

to the Ad capsid through association with the penton base [1]. The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation [2]. By extending the knob away from the virion, the shaft facilitates its interaction with host receptor [1]. The trimeric subunits of the carboxyl (C)-terminal knob domain are responsible for binding to the host's primary cellular receptor [3,4].

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding over 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two early delayed (intermediate) transcription units (pIX and IVa2) and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITR) at the end of the viral genome function as replication origins (Fig. 2). The E1A gene is the first transcription unit to be activated shortly after infection, and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus is propagated in E1-transcomplementing cell lines, such as 293 [5], 911 [6], or PER.C6 cells [7]. The E3 region-encoded proteins modulate the host defense, but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted [8], and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer [9], E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes [8].

The coxsackievirus and adenovirus receptor (CAR), a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A, C, D, E and F [10–12]. Entry of Ad5 into cells is initiated by the attachment of fiber on the surface of the capsid to the CAR on the cell surface. The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid to the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$, helps mediate the internalization of Ad5 into the cell [13–15]. Furthermore, heparan sulfate glycosamino-glycans have also been reported to serve as primary attachment sites for Ad2 and Ad5 [16]. The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base [17]. The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell [18].

Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and nondividing cells. They have large packaging ability for foreign genes and can be easily grown to high titers and purified for clinical applications. Furthermore, Ad is nononcogenic, and Ad-related pathology is mostly limited to mild upper respiratory tract infections. All these advantageous features lead to increasing number

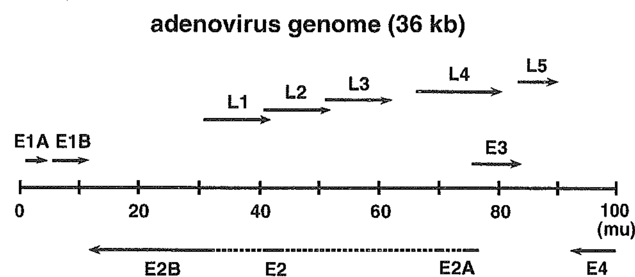

Fig. 2. Genome structure of human Ad serotype 5. The early transcription units E1 (and E3) are deleted for the first-generation Ad vectors.

of clinical protocols employing Ad vectors. As of October of 2003, the percentage of gene therapy protocols utilizing Ad vectors was at 27% (636 protocols) and the percentage of patients treated with Ad vectors at 18% (3496) (Journal of Gene Medicine Website, www.wiley.co.uk/genmed/clinical). This proportion is second only to retroviral vectors.

However, the immune response to the Ad vector-transduced cells dramatically affects the kinetics of 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. Though Ad vectors find niches in the treatment of degenerative diseases like vascular and coronary artery diseases in which transient transgene expression is advantageous [19,20], and for cancer, in which cellular toxicity and immunogenicity might enhance antitumor effects [21], less antigenic Ad vectors with long-term transgene expression are preferable in most cases. Here, we would like to highlight various approaches to overcoming the hurdle of Ad vector immunogenic toxicities to improve the kinetics of Ad vector-delivered genes and gene products.

2. Immunogenic toxicities of Ad vectors

The greatest obstacle for gene therapists wanting to exploit Ad vectors is the issue of their viral immunogenic toxicities. With the extensive efforts of various research groups, more and more data concerning Ad vector immunogenic toxicities have been obtained. However, it is apparent that the immunogenic toxicities associated with the use of Ad vectors are extremely complex, involving both innate and adaptive immune responses, along with apoptosis, and we are still far from a thorough understanding of all the aspects of the toxic interaction between Ad vectors and their hosts.

The complexity of Ad vector immunogenic toxicities lie in the fact that they induce multiple components of the immune response [22–24]. The cytotoxic T-lymphocyte (CTL) response can be elicited against viral gene products and/or transgene products expressed by transduced cells, resulting in a host immune attack against the transduced cells and the elimination of transgene-expressing cells. The Ad capsid itself can induce humoral virus-neutralizing antibody responses, which prevent transgene expression on subsequent administration of vectors of the same serotype, and which also provoke potent cytokine-mediated inflammatory responses during which NF κB activation might play a central role [25,26]. It is thought that among the inflammatory cytokines, TNF-α plays a dominant role in Ad vector clearance [26,27]. Wilson's group demonstrated that systemically administered Ad vectors preferentially activated dentritic cells and macrophages in the spleen to release inflammatory cytokines, independent of transgene expression [28,29]. This suggests in designing targeted Ad vectors, it should be taken into consideration to reduce transduction of or sequestration by antigen presenting cells.

Like all drug-associated toxicities, the degree to which Ad vectors induce harmful immune-mediated and inflammatory responses and other toxic side effects is dose-dependent [30–32]. Highly dangerous inflammatory responses might be inappropriately activated, especially at high doses of Ad vectors. During the tragic 1999 gene therapy trial for deficiency of ornithine transcarbamylase (OTC) at the University of Pennsylvania (U.S.), an escalated dose of Ad vector (3.8×10^{13} particles) systemically administered through the hepatic artery induced a massive systemic inflammatory response that led to fever, disseminated intravascular coagulation, multi-organ failure and the eventual death of an 18-year-old patient [33,34]. Besides the potential of a lethal outcome, immunogenic toxicities attenuate the therapeutic efficacy by affecting the kinetics of delivered genes and gene products.

3. Kinetics of Ad vector-delivered gene and gene product

It is known that different viruses are cleared from the blood stream by Kupffer cells (KC) [35]. However, blood clearance varies among different viruses [35]. Important determinants of virus clearance from the blood stream include interaction between viral components and cellular receptors, and virion size. The net charge of the viral particle also affects the clearance kinetics [36]. Interventions that slow the

clearance of Ad vectors from the blood stream might favor tissue- or tumor-specific targeting approaches based on systemic delivery [37,38]. Understanding the kinetics of blood clearance and biodistribution of Ad vectors would be beneficial to the advance of their application as therapeutic agents.

Several groups have verified that KC played a central role in clearing the Ad particles from the blood stream by blocking the KC of mouse liver [26,39,40]. The Curiel group's as well as our own, indicated rapid clearance of Ad vectors from the blood of mice, with a half-life of less than 3 min and negligible levels of the Ad vectors remaining in the blood 30 min after injection (Fig. 3A) [38,41,42]. To examine the role of the liver in the blood clearance of Ad vectors, we determined the amounts of Ad vector DNA in the mouse. In accordance with the other groups' data [40,43], we found that 98% of the intravenous dose had accumulated in the mouse liver 1 h after injection. Forty-eight hours after injection, 43% of the input Ad5 DNA persisted in the liver (Fig. 3B) [41]. These data confirm that, following intravenous administration, Ad vectors are predominantly delivered to the liver. Besides the strong interaction between viral components and cellular receptors (e.g. the fiber-CAR and RGD motif of the penton base-αv integrins), the inclination of Ad vectors to the mouse liver may also be attributed to the anatomical properties of the liver sinasoid [43,44]. The accumulation of Ad vectors in the liver may itself be toxic. We further investigated the cellular distribution of Ad vector genomes in mouse livers after intravenous injection, and found them to be equally distributed in the parenchymal cells (PC; hepatocytes) and nonparenchymal cells (NPC; Kupffer cells and endothelial cells), when 1.5×10^{10} particle of Ad vectors were intravenously injected into the mouse [41]. Despite the high uptake of Ad vectors by the NPC, the Ad vector-mediated transduction efficiencies in the NPC were much lower than those in the PC, indicating the uptake of Ad vectors by the NPC is a function of phagacytosis rather than a receptor-mediated infectious pathway [41]. This result of the high uptake of Ad vectors by the NPC combined with low transduction efficiencies is consistent with previous reports of nonlinear dose responses of Ad transduction in the liver [45,46]. Those results suggested that there was a viral dose threshold effect for efficient liver transduction of Ad vectors and that NPC played a central role in this threshold effect, such that low doses of Ad vectors were efficiently taken up by the NPC without appreciable transgene expression, while high doses saturated the NPC and were able to productively transduce the PC. Hence, depleting the Kupffer cells or blocking their uptake before Ad vector administration might be helpful in reducing the Ad vector dose for systemic route gene therapy [45,46].

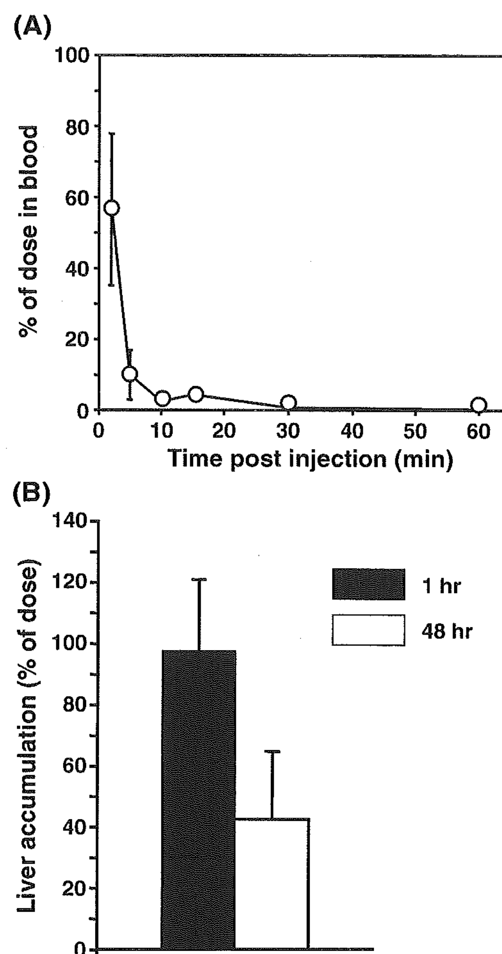

Fig. 3. Blood clearance kinetics and liver accumulation of Ad vectors after intravenous administration into mice. E1- and E3-deleted Ad vectors (1.5×10^{10} particle) were intravenously injected: (A) blood was drawn from the retro-orbital at the indicated times postinjection. (B) The livers were isolated 1 or 48 h after injection. Total DNA, including the Ad vector genome, was isolated from the blood or the livers, and slot-blot analysis was then performed. The data in this figure were published in our previous paper [41].

It is putative that transgene expression from Ad vectors is transient because of the rapid clearance of viral particles by the host immune response. However, duration of transgene expression may vary according to transgene products or animal species/strains. For example, marked variability was observed in the persistence of human alpha 1-antitrypsin (hAAT) expression delivered by an E1/E3-deleted Ad vector in different mouse strains, ranging from several weeks in the strains of C3H/HeJ and Balb/c to more than 3 months in the strains of C57Bl/6, B10.A(2R) and B10.BR [47]. This is because immunogenicity varies according to different transgene proteins, and immune responses (cellular and/or humoral) to invading virions vary according to different species or strains [48].

Overall, the kinetic features of Ad vector-delivered genes and gene products might be summarized as rapid clearance of virus from blood, liver accumulation of virus DNA and expression, and transient transgene expression. Moreover, unlike commonsense pharmaceuticals, readministration of Ad vectors is problematic due to neutralizing antibodies.

4. Approaches to improving the kinetics of Ad-delivered genes and gene products

The disadvantageous kinetics of Ad vector-delivered genes and gene products for gene therapy applications results from their immunogenic toxicities. All the potential approaches to improving the kinetics should be based on attenuating the immunologic interaction between Ad vectors and hosts, so as to extend the persistence of the virus in the blood, reduce the accumulation of the virus and transgene expression in the liver, prolong circulational or local transgene expression in the organ/tissue of interest, and make readministration possible.

4.1. Viral genome deletion

Ad vectors have been extensively engineered to reduce their immunogenicity. First-generation Ad vectors were deleted for only one or two viral early genes (E1 and E3). Cells transduced with these vectors expressed other Ad genes at low levels, inducing strong cytotoxic T-cell responses that rapidly eliminated transgene expression. Second-generation vectors that contain additional deletions in other early genes (E2 and/or E4) have shown reduced toxicity profiles compared to first-generation Ad vectors due to the decreased Ad protein synthesis in transduced cells [49–51]. However, the remaining viral gene expression still induces the T-cell response, which is difficult to overcome.

Progress has been made in reducing T-cell responses against viral gene products expressed by transduced cells, by engineering "helper-dependent" (HD) or "gutless" or "gutted" Ad vectors, from which all viral genes are deleted except the inverted terminal repeats (ITR) sequences at the two ends and the packaging signal of the Ad genome. The HD Ad vectors are produced with a helper Ad that provides in-trans the necessary viral proteins required for replication and packaging of the HD vector (Fig. 4). This advance has improved the prospects of Ad vectors for long-term gene transfer [52]. Several application experiments have shown that the HD Ad vectors have facilitated life-long phenotypic correction in mouse models with negligible toxicity. For example, in a mouse model of hyperlipidemia, a defect correction was observed for 2.5 years with a single injection of an HD Ad vector [53]. In another mouse model of hemophilia, expression of human factor VIII was sustained for longer than 9 months [54]. However, in canine models of hemophilia A or B, only transient phenotypic correction, and in some of those cases only partial correction, was observed with no detectable toxicity using an HD Ad vector [55,56], while the same group of researchers achieved long-term phenotypic correction in a mouse hemophilia B model without toxicity by a single injection of an HD Ad vector [57]. Though the discrepancy of transgene persistence by HD Ad vectors between mouse and large animal models still needs to be elucidated, it is clear that immunogenic toxicities induced by HD Ad vectors are greatly reduced.

Compared with early generation Ad vectors, yields of HD Ad vector production need to be increased, and caution should be taken to decontaminate helper Ad. Some advances have been achieved in these aspects. Sakhuja et al. [58] developed an optimized HD Ad vector production system by generating a novel producer cell line, PERC6-Cre, which was adapted to serum-free suspension culture for bioreactor mass

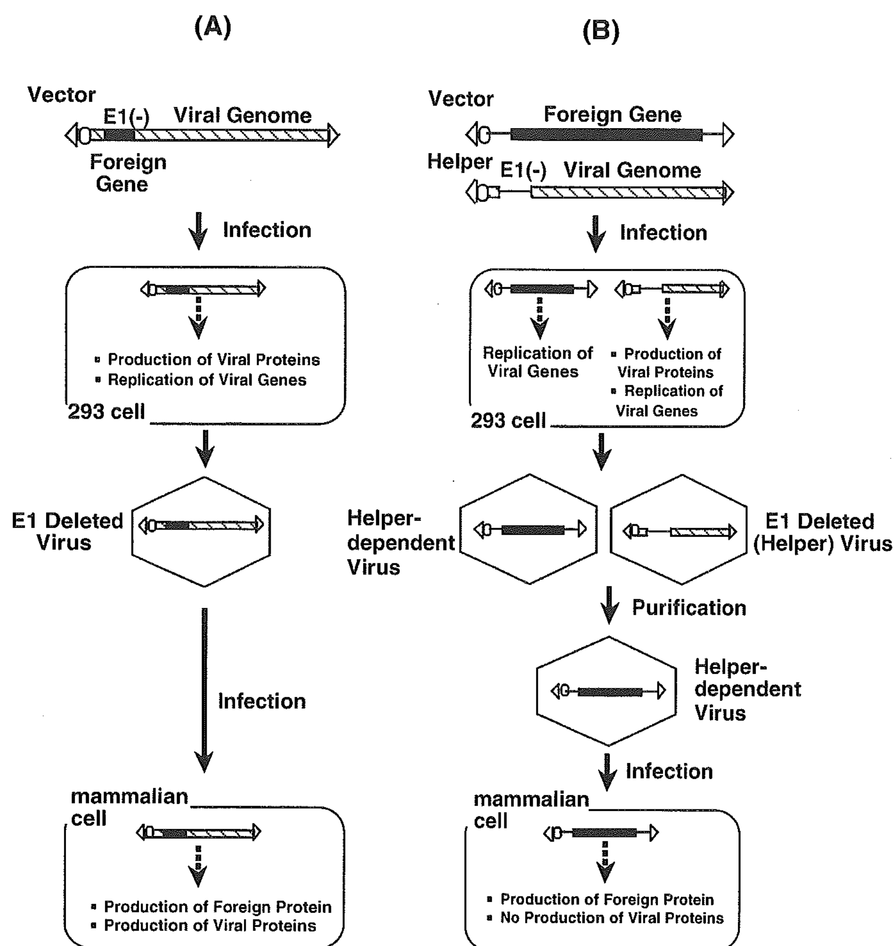

Fig. 4. Propagation diagrams of the first-generation Ad and helper-dependent Ad vectors. (A) The first-generation Ad vectors are produced in E1-transcomplementing cell lines, such as 293, 911, or PER.C6 cells. (B) Helper-dependent (HD) Ad viruses are propagated with the viral proteins provided by helper Ad viruses. To reduce the production of the helper virus, a Cre/loxP recombination system is generally utilized to excise the packaging signal from the helper virus genome [59].

production of HD Ad vectors. However, they also indicated that using the existing Cre/loxP technology to excise the packaging signal from the helper virus genome, which was originally developed by Graham and colleagues [59], could not completely eliminate the helper virus from HD Ad preparations.

With the advantages of reduced toxicity, larger packaging capacity for foreign genes of up to 36 kb, and possible persistent transgene expression, HD Ad vectors remain a powerful tool for gene therapy, though the humoral response against incoming capsid proteins shortly after administration remains a major challenge.

4.2. Modulation of the viral tropism

The broad tropism of Ad, on one hand, leads to unwanted vector uptake by many different cell types in multiple organs when the vectors are delivered systemically. Even the local delivery of Ad vectors can lead to leakage and dissemination to other tissue, resulting in toxic effects on distal sites, most notably the liver [60–62]. On the other hand, important types of target tissues are refractory to Ad infection due to CAR scarcity; these include primary tumor cells [63–66], mature skeletal muscle [67], endothelial [68,69], smooth muscle [68,69], differentiated airway epithe-

lial [70–72], lymphocytes [69,73,74], fibroblasts [68,69,75], hematopoietic cells [76] and monocyte-derived dendritic cells [77,78] and require an escalating dose of vector in order to achieve efficient gene transfer. This in turn increases vector-associated immunogenic toxicities. Hence, lack of Ad vector specificity is directly linked to the induction of massive systemic immune responses. Furthermore, localizing gene transfer by Ad vectors to specific cell types is likely to reduce immunogenic toxicities by allowing lower does to be administered. Therefore, there is a strong rationale for the development of tropism-modulated Ad vectors of enhanced specificity and gene transfer efficiency. In recent years, there have been significant efforts to improve Ad transduction efficiency to targets that are resistant to Ad infection due to CAR deficiency [79,80]. Ad vectors with the native tropism completely ablated have also been successfully developed [42,81].

4.2.1. Increasing transduction of target cells by bispecific conjugates

Douglas et al. [82] first reported the bispecific conjugate-based approach. They conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and was shown to redirect the Ad infection of target cells via the folate receptor at a high efficiency. Furthermore, when complexed with an Ad vector carrying the gene for herpes simplex virus thymidine kinase, the Fab–folate conjugate mediated the specific killing of cells that overexpress the folate receptor [82]. After that, the Fab fragment of the anti-fiber monoclonal antibody has been utilized to conjugate with several other ligands. For example, the Fab has been conjugated with (1) basic fibroblast growth factor (FGF2) to target various cells [83], including Kaposi's sarcoma cell lines [84], and ovarian cancer cells [85–87]; (2) mAb against the epidermal growth factor receptor (EGFR) to target glioma cells [64] and squamous cell carcinoma [88]; (3) an anti-CD40 mAb fragment to target dendritic cells [77]; (4) anti-angiotensin converting enzyme (ACE) mAb to target pulmonary endothelial cells [89]; (5) and an Hc fragment of tetanus toxin to target neuronal cells [90]. Theoretically, in this approach, any conjugates with one component directed against the Ad capsid and the second component directed against the cell surface protein can be applied to increase transduction of the target cells. The component directed against the Ad capsid can be the neutralizing Fab fragment of an anti-fiber monoclonal antibody as described above, a neutralizing anti-Ad knob single-chain antibody (scFV) [91–95] or the extracellular domain of CAR [96,97]. The targeting cell-binding moiety can either be natural molecules or man-made peptides identified by phage display technique [92,98,99].

4.2.2. Increasing transduction of target cells by genetic modification of the fiber

Since the fiber stretches out from the capsid and plays a central role for Ad binding to the native receptors, many attempts have focused on genetic modifications of the fiber. This approach can be divided into two main sub-approaches.

One is to incorporate ligands into the fiber knob. In order not to destroy the fiber trimerization, and to facilitate the ligands to access their cognate receptor, the HI loop and C-terminal of the Ad fiber knob have been found to be most appropriate to accommodate the foreign ligands [100,101]. We and other groups showed that Ad vectors containing the RGD motif in the HI loop greatly increased by as much as 3 orders the efficiency of gene delivery to a variety of CAR-deficient cells including primary and established ovarian cancer cells [102,103], squamous cell carcinoma [104], leukemia [105,106], rhabdomyosarcoma [107], dendritic cells [108,109], glioma [94,105], pancreatic cancer cells and primary human endothelia cells [103]. These results indicate that the integrin family could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by RGD-modified Ad vectors. Currently, the RGD-modified Ad vector is being tested in a phase I clinical trial of ovarian cancer and recurrent cancer of the oral cavity and oropharynx [80]. Besides the RGD motif, there are reports of inserting the peptide SIGYPLP (Ser-Ile-Gly-Tyr-Pro-Leu-Pro), which was discovered by phage display to show high affinity to vascular endothelial cells [92], in the HI loop to increase transduction of vascular endothelial cells [110] and cancer cells [111]. In terms of the incorporation of foreign peptide into the C-terminal of fiber knob, we and another group found that a peptide containing seven lysine residues could be

inserted to increase transduction efficiency to a variety of CAR-deficient cells [69,112].

Another sub-approach is fiber-pseudotyping. Since Ads that belong to subgroup B, such as Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50, recognize CD46 as the primary cellular receptors [113,114], fiber (knob, or knob and shaft) substitution could alter the tropism of Ad5 vectors. This strategy was first reported by Gall et al. [115]. They constructed a chimeric Ad5 vector by replacing the Ad5 fiber gene with the fiber gene from Ad7 (although Ad7 belongs to subgroup B, its receptor has not been identified), and found altered tropism to Ad5 vectors. Shayakhmetov et al. [76] constructed an Ad5 vector with a chimeric fiber (Ad5 tail/Ad35 shaft/Ad35 knob) and showed increased transduction of CD34+ cells relative to the unmodified Ad5 vector. Also, the same group incorporated the Ad11 fiber to Ad5 and found enhanced infection of human hematopoietic progenitor cells [116]. An Ad5-based vector containing the Ad16 fiber shaft and knob domains yielded an 8- and 64-fold increase in gene transfer to endothelial and smooth muscle cells as compared to Ad5 [117] and an 150-fold increase in gene transfer to cultured synoviocytes as compared to Ad5 [118]. Replacement of only the knob domain of the fiber can also alter viral tropism. Stevenson et al. [119] demonstrated that replacement of the Ad5 fiber knob with the Ad3 fiber knob (which, though it also belongs to subgroup B, has been shown not to use CD46 as a high-affinity attachment receptor [113,114,120]) improved gene delivery to human fibroblasts and head and neck cancer cells when compared to unmodified Ad5. Takayama et al. [121] generated a dual-knob mosaic Ad virus by incorporating both Ad5 and Ad3 knobs in the same particle, which displayed infectivity enhancement and tropism expansion by utilizing either receptor, CAR or the Ad3 receptor, for virus attachment to cells.

In addition to the approaches at the level of transduction, increasing transduction of the target cells can also be achieved to some extent at the level of transcription by using cell-specific promoters. Furthermore, combining transductional and transcriptional targeting seems to be an attractive strategy to enhance the targeting effect of Ad vectors. Reynolds et al. [122] reported that in an Ad vector, the combination of transductional targeting by linking the Fab fragment of an anti-Ad5 knob antibody to the anti-ACE (pulmonary endothelial marker) monoclonal antibody mAb, and an endothelial-specific promoter (flt-1) resulted in a synergistic, 300,000-fold improvement in the selectivity of transgene expression for the lung versus the usual site of vector sequestration, the liver. Barnett et al. [123] obtained great synergistic targeting effect in cancer cells using a similar dual targeting strategy with the target molecule being epidermal growth factor receptor (EGFR), which is overexpressed on many tumor cells; the specific promoter was the osteocalcin gene 2 promoter, which has specificity for osteoblasts and osteoblastic metastatic lesions. Nicklin et al. [111] also observed the synergistic targeting effect in certain cancer cells by combining transductional targeting (incorporating the SIGYPLP peptide into the fiber knob) with transcriptional targeting (via the FLT-1 promoter).

4.2.3. Ablation of the native tropism

Though the above tropism-modified Ad vectors could greatly improve transduction efficiency to many CAR-deficiency cells, when systemically administered, sometimes vector dissemination, resulting in liver accumulation, is still unavoidable. To create a strictly targeted Ad vector, two basic requirements are thought to be necessary: interaction of Ad with its native receptors must be completely removed and novel tissue-specific ligands must be added to the virus capsid (Fig. 5).

The capsid proteins determine the tropism of Ad. The fact that Ad5 uses multiple receptors such as CAR, αv integrin and heparan sulfate to transduce various cells implies that the Ad5 capsid must be multi-engineered to abolish its native tropism. Several groups including us have shown that vectors with the ablation of only CAR-binding, i.e., vectors in which the AB, DE, or FG loop of the fiber knob was mutated, do not change the systemic gene-transfer properties [124–127]. Vectors with the ablation of only αv-integrin-binding also show similar or slightly decreased liver transduction compared with wild-type Ad vectors [126]. Furthermore, the length [128–130] and the KKTK motif of the fiber shaft [81] have been reported to influence Ad5-mediated in vivo gene transfer. We supposed that Ad5 tropism would be determined by at least three factors: the fiber knob, the fiber shaft and the RGD motif at the penton base. Thereby, we developed a triple-mutant Ad5 vector by

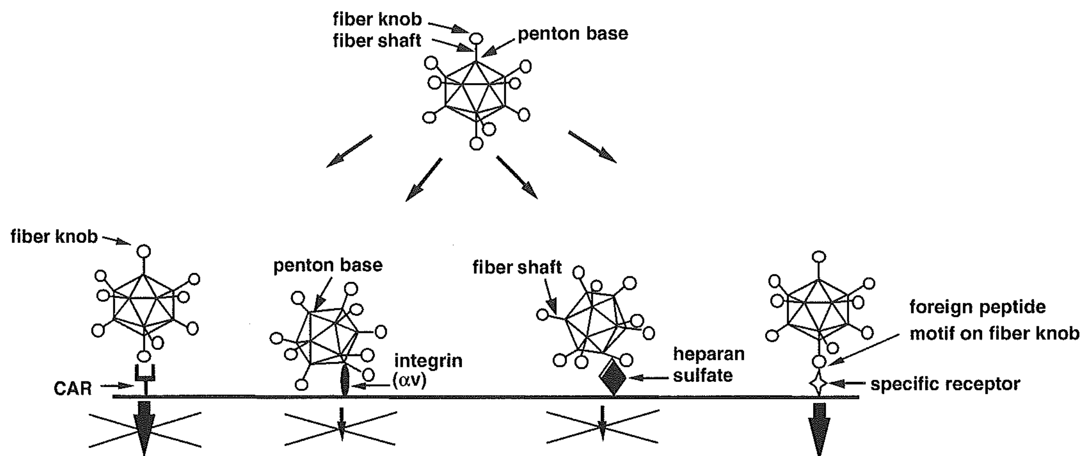

Fig. 5. Schematic diagram of targeted Ad vectors with the original tropism ablated. With the triple mutations in the fiber knob, fiber shaft and penton base, the CAR-, αv intergrin- and heparan sulfate-binding activities of the Ad viral capsid are completely ablated. Targeting effects can be achieved by either the bispecific conjugate or the foreign ligands incorporated into the fiber knob.

(1) mutating the fiber knob to ablate CAR-binding interaction, (2) replacing the Ad5 shaft with a shorter shaft from Ad35, which contains no KKTK motif to ablate binding with heparan sulfate, and (3) depleting the RGD motif at the penton base to ablate αv-integrin-binding interaction. As expected, this triple-capsid-mutant Ad5 vector exhibited little tropism to any organs (Fig. 6). Compared with the wild-type Ad5 vector, it showed 30,000-fold lower mouse liver transduction [42]. This indicates that to ablate the original tropism of Ad vectors, all three parts of the capsid (fiber knob, shaft and penton base) associated with the original tropism should be simultaneously blocked. Smith et al. [81] utilized a similar strategy and reached the same conclusion as ours.

Due to the multiple mutation of the capsid, the triple-mutant Ad vectors could not be produced by the original protocol using 293 cells. To support the propagation of our triple-mutant Ad5 vector, we generated a mutant 293 cell line stably expressing wild-type Ad5 fiber protein (Fiber-293 cells). It is possible to produce this mutant Ad vector to high titer using Fiber-293 cells. Furthermore, for the convenient display of foreign ligands, both the HI loop and C-terminal region of the fiber knob and the region of the RGD motif of the penton base were designed to have unique restriction sites. Therefore, by using our simple in vitro ligation method, the targeting ligands can easily be displayed in the capsid of our triple-mutation Ad vector [42]. We suggest that our triple-mutation Ad vector provide for a platform for future targeted Ad vector development. Future efforts should be directed into exploring novel ligands for specific tissue targeting.

Our triple-mutant Ad vector described here should be easily combined with other approaches such as transductional targeting, transcriptional targeting and even deletion of viral genomes to create less immunogenic vectors. Such combination will no

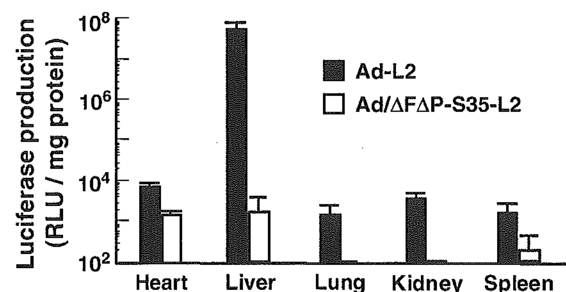

Fig. 6. Biodistribution of a transgene product (luciferase) in mice after the systemic administration of Ad-L2 (conventional Ad vectors) or Ad/ΔFΔP-S35-L2 (with triple mutations in the regions of fiber knob, fiber shaft and penton base). Ad-L2 or Ad/ΔFΔP-S35-L2 (3.0×10^{10} VP) were intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean±S.E. of five mice. The data in this figure were published in our previous paper [42].

doubt improve the kinetics of Ad vectors to better meet the requirements of clinical trial.

4.3. Cationic liposome complexation or PEGylation

The most straightforward approach for circumventing Ad-induced immunogenic toxicities has been the use of cationic liposome and polyethylene glycol (PEG) to shield the vectors from the host immune system.

Cationic liposomes have shown promise as a gene delivery tool for plasmid DNA due to their simplicity, safety and efficiency in some tissues [131]. Several groups have observed that cationic liposome-conjugated Ad vectors greatly increase gene transduction efficiency to a variety of cell and tissue types that are especially resistant to Ad infection, including vascular tissue [132], human smooth muscle cells [133], airway epithelia [134] and human glioma cells [135]. Fasbender et al. [134] suggested that viral binding was dependent on an electrostatic interaction with the cell surface, that viral entry did not require an interaction of the Ad fiber protein with the cell surface, and that cationic liposome-conjugated Ad vectors entered cells via a pathway different from that utilized by Ad alone. Use of a more efficient delivery system could allow a smaller dose of Ad vector to be administered for therapeutic effects, thereby decreasing the total immune response. Yoshida's group noted that mice injected with cationic liposome-conjugated Ad vectors produced fewer anti-Ad antibodies compared with an equivalent dose of unconjugated Ad vectors, and cationic liposome-conjugated Ad vectors were less susceptible to inactivation by neutralizing antibodies than Ad vectors alone [136,137]. The increased transduction efficiency, reduced antigenicity, and attenuated susceptibility to neutralizing antibodies might be beneficially multiplied for redosing.

Complexation with PEG (PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase blood persistence of therapeutic proteins such as erythropoietin (EPO), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) etc. [138–140]. Covalent attachment of PEG to the surface of the Ad vector might prolong persistence in the blood and circumvent neutralization of Ad vectors by antibodies [141]. There is a report that PEGylated Ad vectors exhibited a 4-fold slower clearance rate than the non-PEGylated Ad vectors [38]. Furthermore, coupling of PEG to the viral capsids attenuates the ability of the vector to infect antigen presenting cells, thereby reducing inflammatory responses. Animals administered with the PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression upon readministration of unmodified Ad vectors in the lung [141,142]. However, PEGylation might lead to loss of infectivity [38]. The activated PEG reacts preferentially with the ε-amino terminal of lysine residues on the capsid, including the hexon, fiber and penton base, which might lead to decreased infectivity. Experimental optimization of the PEGylation reaction so that PEG occupies only 70 to 80% of the available sites on virus capsid proteins shielded vectors from antibody neutralization and retained viral infectivity [143,144]. Nonetheless, compared with local readministration, the results of systemic readministration of the PEGylated Ad vectors were less encouraging. Levels of transgene expression achieved by systemic readministration of the PEGylated Ad vectors were on the order of 1000- to 10,000-fold lower than that seen in animals carrying no neutralizing antibodies against Ad; these levels were also significantly lower than those observed with readministration of these modified vectors in the lung [142]. Thus, the exact nature of the immune response against the PEG modified Ad must be characterized further.

4.4. Immune intervention

The success of long-term gene therapy by Ad vectors depends on finding ways to avoid/attenuate the induction of immune responses to both the vector and the transgene product. Some advances have been made in the development of novel strategies to disrupt or modulate immune responses in various animal models; these approaches include inhibiting cell-mediated immune responses to prolong transgene expression, and inhibiting humoral immune responses to permit readministration of the vector.

One straightforward approach is to disrupt the inflammatory immune response by inhibition of NF κB activation, thereby blocking release of inflammatory cytokines like TNF-α and IL-6 [145,146]; other relatively simple approaches include pretreatment by