with a four-amino acid mutation of the AB loop (R412S, A415G, E416G, and K417G), the Ad type 35 fiber shaft, and a deletion of the RGD motif of the penton base. Ad/ΔF(FG)ΔP-S35-L2, which is identical to Ad/ΔFΔP-S35-L2 in our previous report (Koizumi *et al.*, 2003a), contains the Ad type 5 fiber knob with a four-amino acid deletion of the FG loop (T489, A490, Y491, and T492), the Ad type 35 fiber shaft, and deletion of the RGD motif of the penton base. Ad/ΔF(AB)ΔP-S35-RGD-L2 contains an RGD motif in the HI loop of the fiber knob in Ad/ΔF(AB)ΔP-S35-L2. Ad-L2 is a conventional Ad vector. All mutations of the mutant Ad vectors and possible interaction of each virus with the cells are summarized in Table 1 and Fig. 1A. All of the mutant Ad vectors used in this study were readily propagated with particle titers similar to that of the conventional Ad vector, Ad-L2 (see Materials and Methods).

To confirm the modification of the fiber protein in each Ad vector, Western blot analysis against fiber protein was performed with rabbit fiber knob polyclonal antibody (Fig. 1B). The mutant fiber and wild-type fiber are easily distinguished because the mutant fiber is smaller than the wild-type fiber because of the small size of the Ad type 35 fiber shaft and because Ad/ΔF(AB)ΔP-S35-L2 has a fiber protein four amino acids longer than that of Ad/ΔF(FG)ΔP-S35-L2. Western blot analysis shows the expected size of the fiber proteins, suggesting that each Ad vector should indeed contain the expected fiber protein.

Gene transfer in vitro

We examined the gene transfer activity in SK HEP-1 cells transduced with Ad/ΔF(AB)ΔP-S35-L2 in comparison with

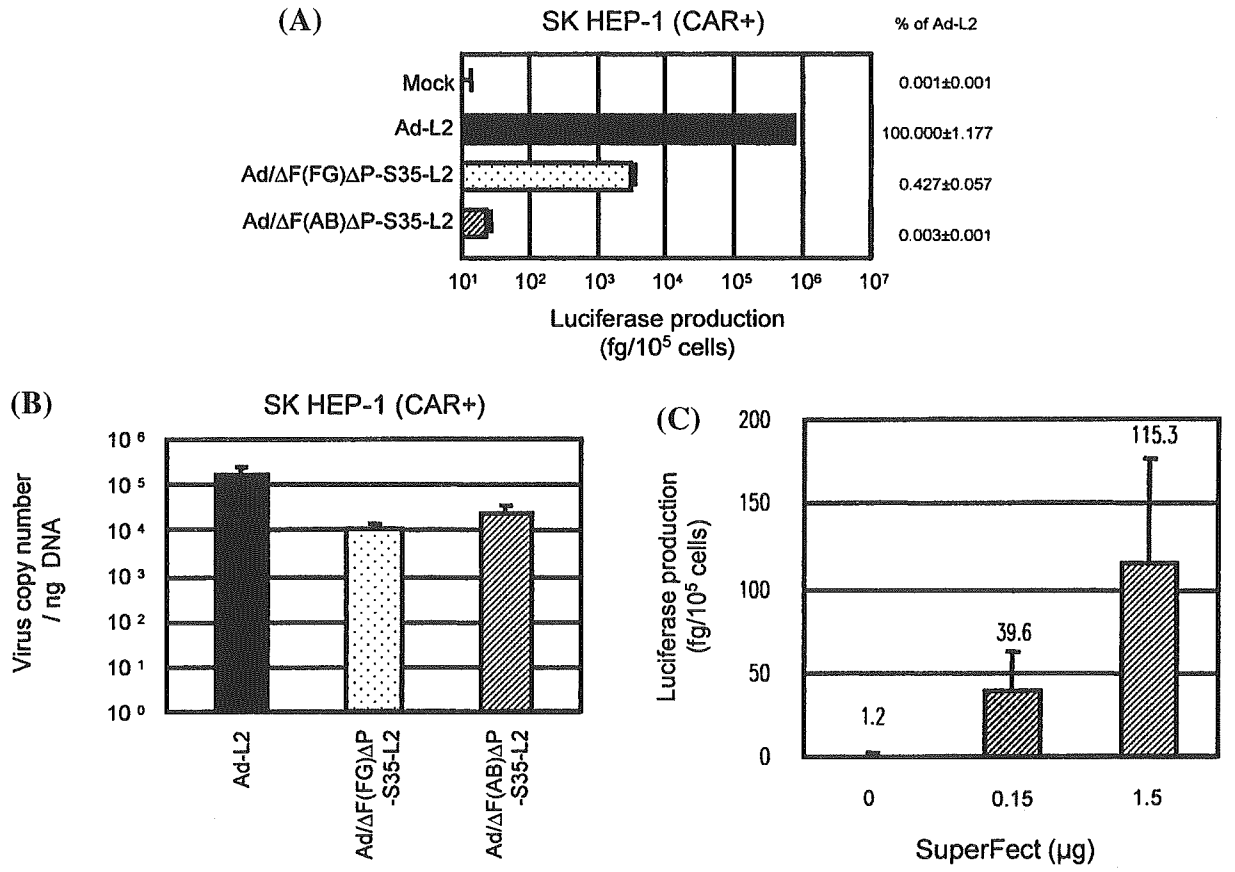

FIG. 2. Luciferase production and viral uptake in SK HEP-1 cells transduced with several Ad vectors. (**A**) Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. SK HEP-1 cells were transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 (3000 VP/cell) for 1.5 hr. After culture for 48 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means ± SD (n = 4). Relative levels of luciferase expression are described by designating the value of Ad-L2 as 100. (**B**) Viral uptake in SK HEP-1 cells. SK HEP-1 cells were transduced with Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 at 3000 VP/cell. After culture for 1.5 hr, the cells were washed with PBS, resuspended in 0.05% trypsin–0.5 m*M* EDTA–PBS solution, and incubated at 37°C for 10 min. After this incubation, the cells were incubated at 37°C for 10 min with 0.05% DNase I–0.5 *M* $MgCl_2$–PBS, washed with PBS, and resuspended in 0.1 *M* EDTA–PBS solution. The amounts of Ad genome DNA isolated from the cells were quantified with the TaqMan fluorogenic detection system. Data are expressed as means ± SD (n = 4). (**C**) Comparison of luciferase production in SK HEP-1 cells transduced with a complex of Ad/ΔF(AB)ΔP-S35-L2 and SuperFect. SK HEP-1 cells (2×10^4 cells) were seeded into a 24-well dish. The next day, the cells were either not transduced or were transduced with a complex of Ad/ΔF(AB)ΔP-S35-L2 and SuperFect (0.15 or 1.5 μg) (Qiagen) for 1.5 hr. After culture for 48 hr, luciferase production in the cells was measured with a luciferase assay system. Data are expressed as means ± SD (n = 4).

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.

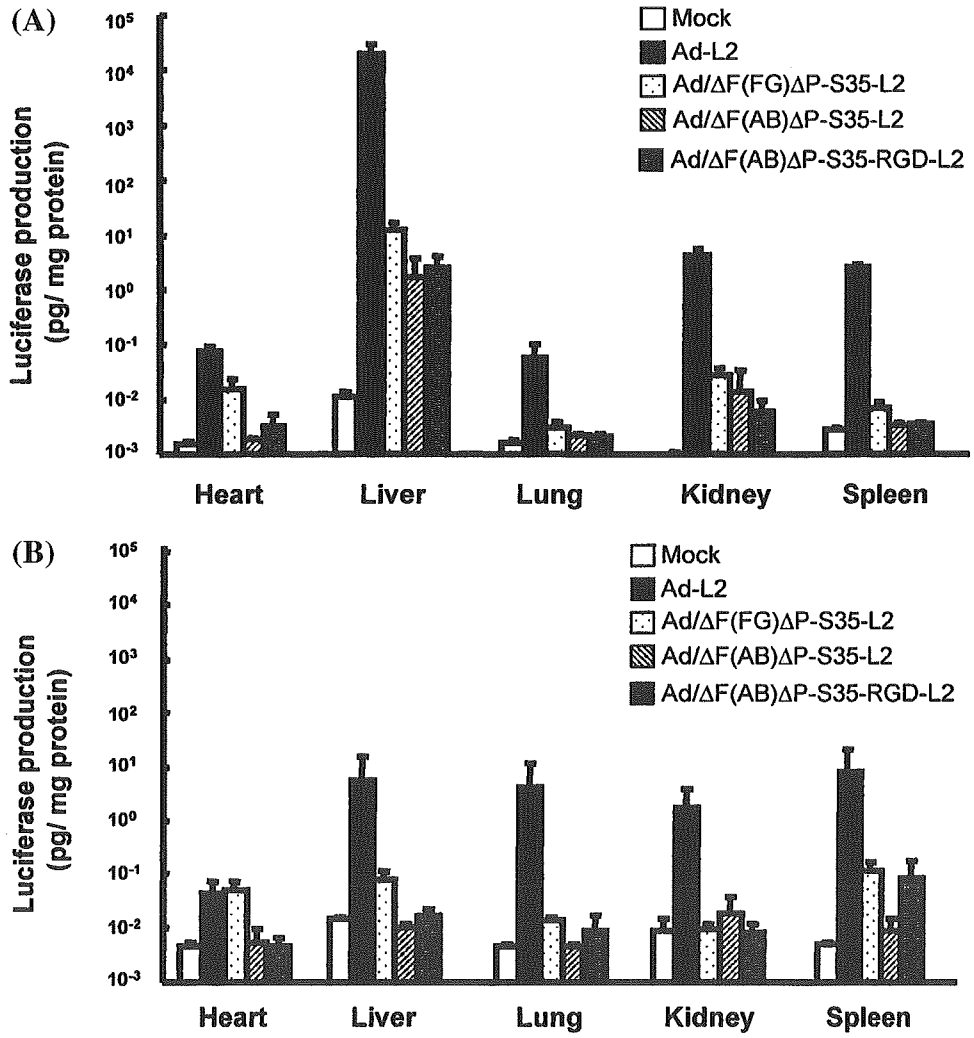

FIG. 3. Luciferase production in mice after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} 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).

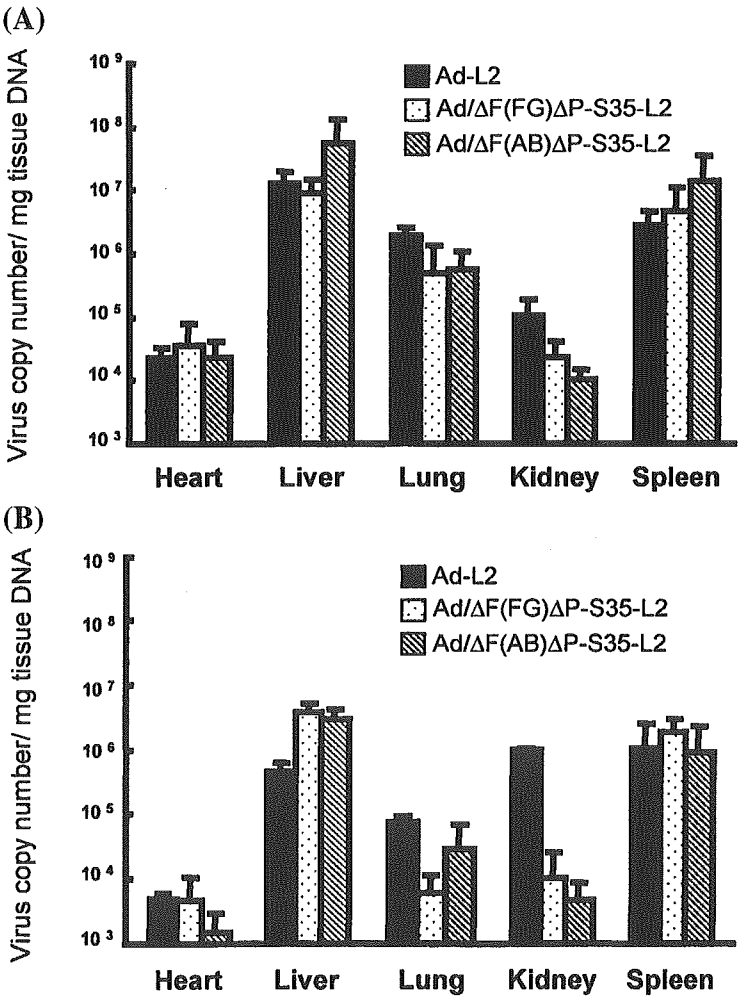

FIG. 4. Biodistribution of viral DNA after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected into mice. Three hours later, 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.

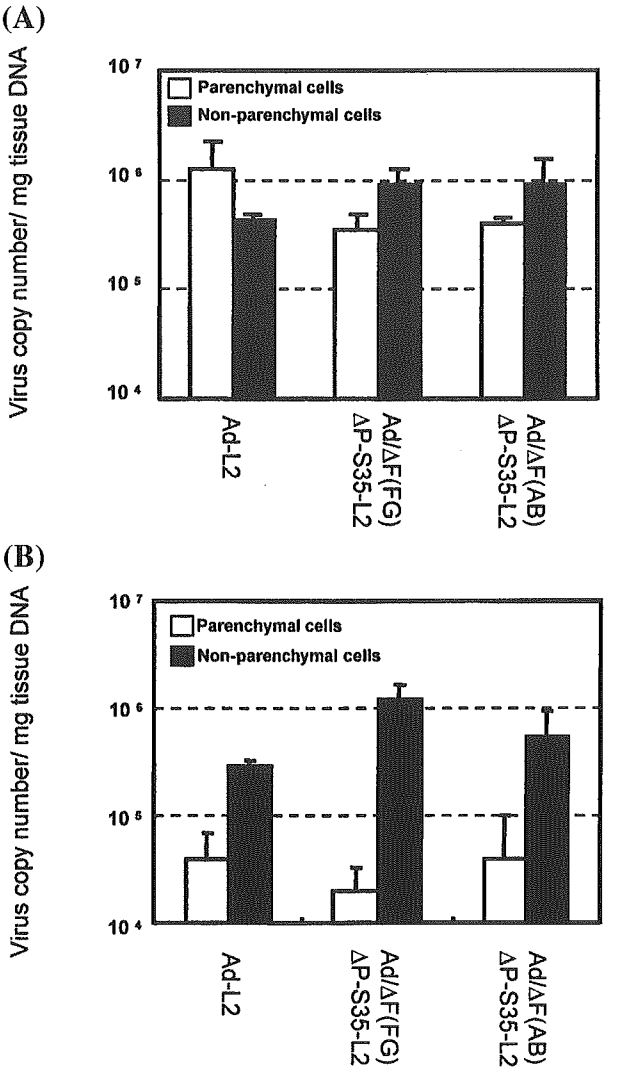

FIG. 5. Biodistribution of viral DNA in liver parenchymal and nonparenchymal cells. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected into mice. Collagenase perfusion was performed 3 hr after injection of 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.

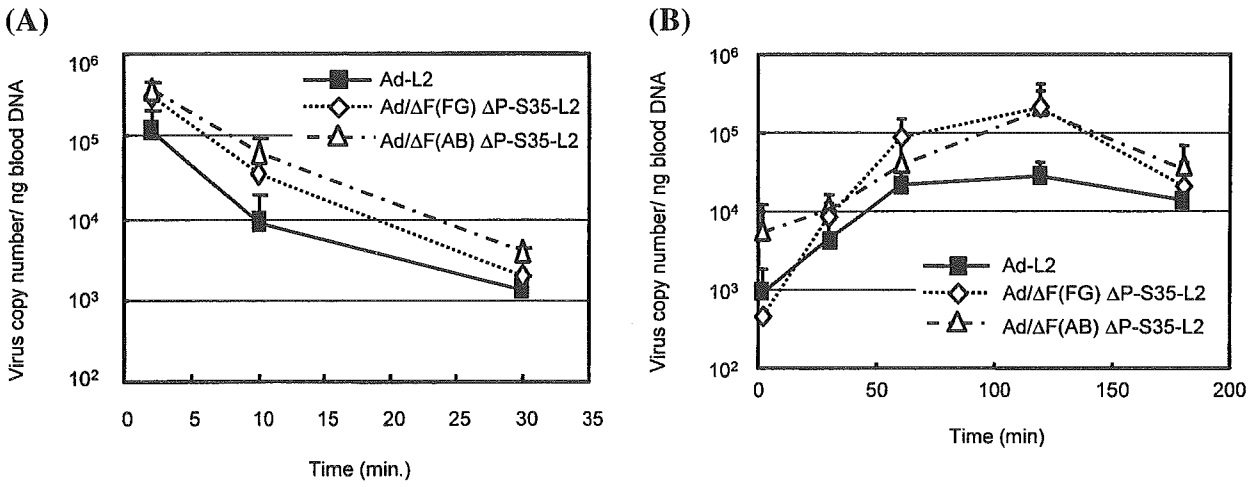

FIG. 6. Blood clearance of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, and Ad/ΔF(AB)ΔP-S35-L2 after systemic administration into mice. Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 was (**A**) intravenously (3.0×10^{10} VP) or (**B**) intraperitoneally (1.0×10^{11} VP) injected, and blood was drawn by retroorbital 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.

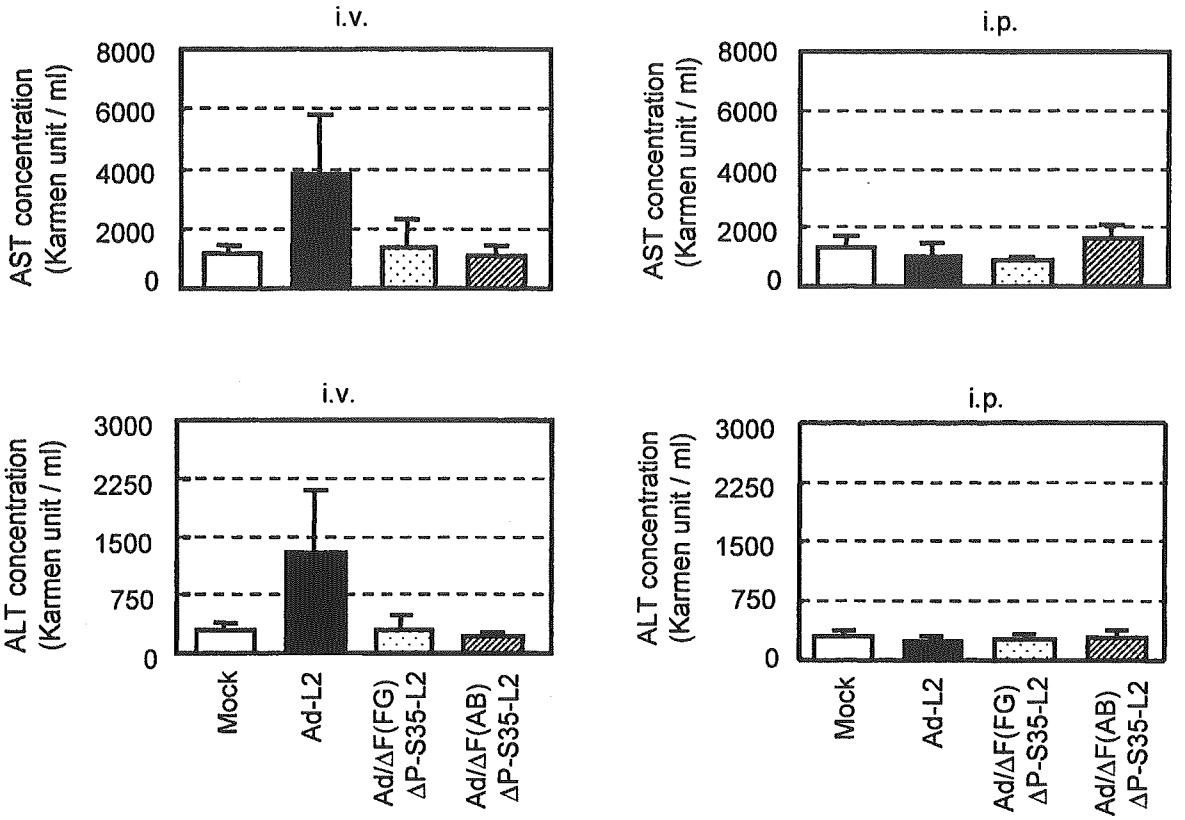

FIG. 7. Serum enzymes levels after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Blood samples were collected from the inferior vena cava 48 hr after intravenous (3.0×10^{11} VP) or intraperitoneal (1.0×10^{11} VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2. 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,

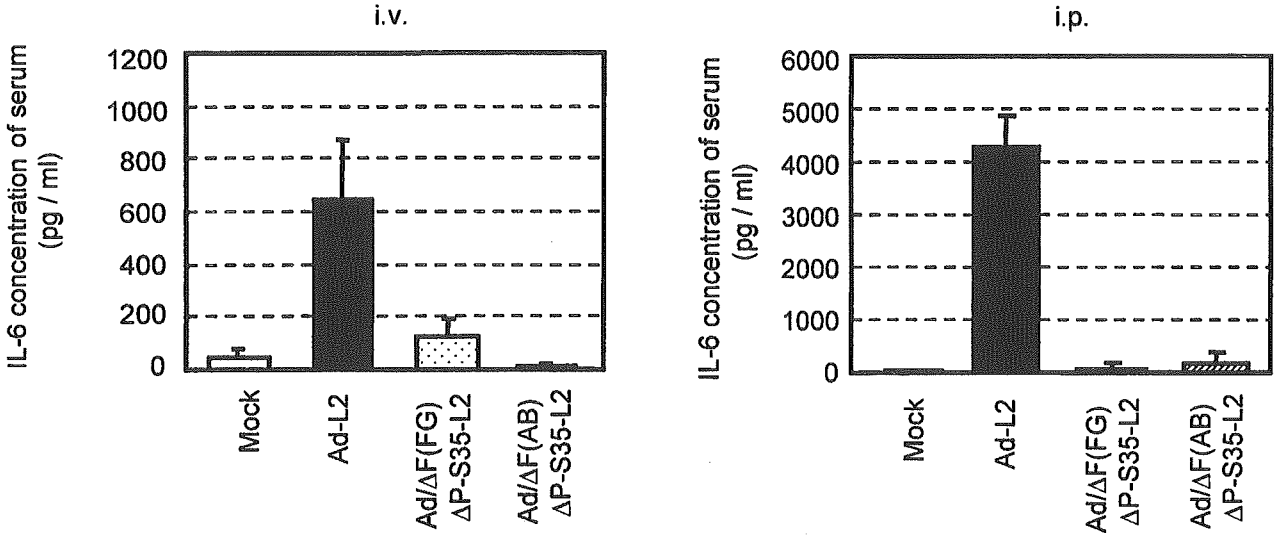

FIG. 8. Interleukin (IL)-6 levels in serum after systemic administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-L2 into mice. Blood samples were collected from the inferior vena cava 3 hr after intravenous (3.0×10^{11} VP) or intraperitoneal (1.0×10^{11} VP) injection of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, 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

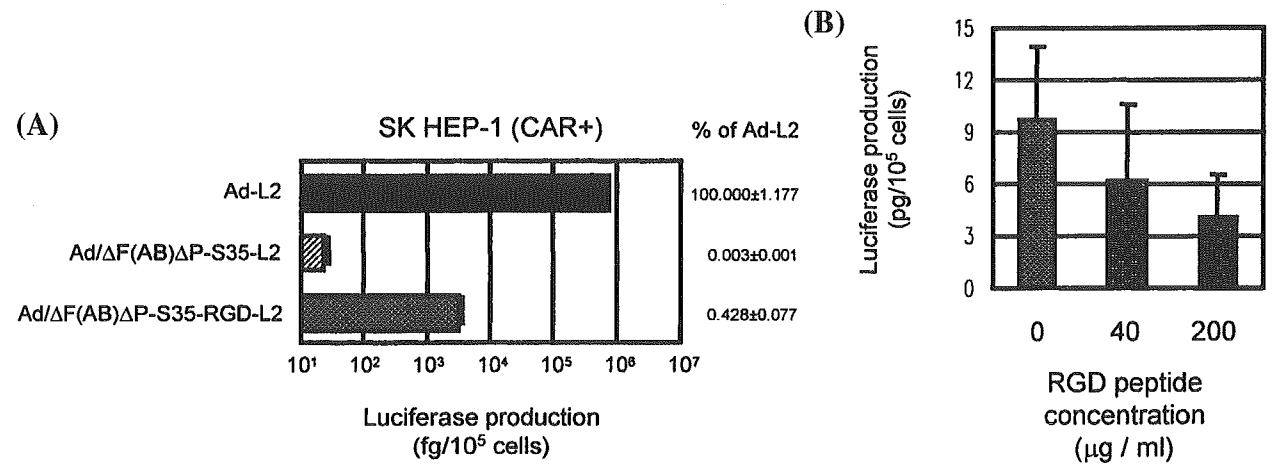

FIG. 9. Luciferase production in human cells transduced with Ad vectors containing RGD motif in the fiber knob. (**A**) Comparison of luciferase production in human cells transduced with Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, or Ad/ΔF(AB)ΔP-S35-RGD-L2. SK HEP-1 cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(AB)ΔP-S35-L2, 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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REFERENCES

AKIYAMA, M., THORNE, S., KIRN, D., ROELVINK, P., EINFELD, D., KING, C., and WICKHAM, T. (2004). Ablating CAR and integrin binding in adenovirus vectors reduces nontarget organ transduction and permits sustained bloodstream persistence following intraperitoneal administration. Mol. Ther. **9,** 218–230.

ALEMANY, R., and CURIEL, D.T. (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther. **8,** 1347–1353.

ALEMANY, R., SUZUKI, K., and CURIEL, D.T. (2000). Blood clearance rates of adenovirus type 5 in mice. J. Gen. Virol. **81,** 2605–2609.

ASAOKA, K., TADA, M., SAWAMURA, Y., IKEDA, J., and ABE, H. (2000). Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the coxsackievirus and adenovirus receptor. J. Neurosurg. **92,** 1002–1008.

BERGELSON, J.M., CUNNINGHAM, J.A., DROGUETT, G., KURT-JONES, E.A., KRITHIVAS, A., HONG, J.S., HORWITZ, M.S., CROWELL, R.L., and FINBERG, R.W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science **275,** 1320–1323.

BEWLEY, M.C., SPRINGER, K., ZHANG, Y.B., FREIMUTH, P., and FLANAGAN, J.M. (1999). Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. Science **286,** 1579–1583.

DE GEEST, B., SNOEYS, J., VAN LINTHOUT, S., LIEVENS, J., and COLLEN, D. (2005). Elimination of innate immune responses and liver inflammation by PEGylation of adenoviral vectors and methylprednisolone. Hum. Gene Ther. **16,** 1439–1451.

EINFELD, D.A., SCHROEDER, R., ROELVINK, P.W., LIZONOVA, A., KING, C.R., KOVESDI, I., and WICKHAM, T.J. (2001). Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. J. Virol. **75,** 11284–11291.

FECHNER, H., HAACK, A., WANG, H., WANG, X., EIZEMA, K., PAUSCHINGER, M., SCHOEMAKER, R., VEGHEL, R., HOUTSMULLER, A., SCHULTHEISS, H.P., LAMERS, J., and POLLER, W. (1999). Expression of coxsackie adenovirus receptor and α_v-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. Gene Ther. **6,** 1520–1535.

HEFFELFINGER, S.C., HAWKINS, H.H., BARRISH, J., TAYLOR, L., and DARLINGTON, G.J. (1992). SK HEP-1: A human cell line of endothelial origin. In Vitro Cell Dev. Biol. **28A,** 136–142.

HONG, S., MAGNUSSON, M., HENNING, P., LINDHOLM, L., and BOULANGER, P. (2003). Adenovirus stripping: A versatile method to generate adenovirus vectors with new cell target specificity. Mol. Ther. **1,** 692–699.

KIRBY, I., DAVISON, E., BEAVIL, A., SOH, C., WICKHAM, T., ROELVINK, P., KOVESDI, I., SUTTON, B., and SANTIS, G. (1999). Mutations in the DG loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. J. Virol. **11,** 9508–9514.

KOIVUNEN, E., WANG, B., and RUOSLAHTI, E. (1995). Phage libraries displaying cyclic peptides with different ring sizes: Ligand specificities of the RGD-directed integrins. Biotechnology **13,** 265–270.

KOIZUMI, N., MIZUGUCHI, H., HOSONO, T., ISHII-WATABE, A., UCHIDA, E., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2001). Efficient gene transfer by fiber-mutant adenoviral vectors containing RGD peptide. Biochim. Biophys. Acta **1568,** 13–20.

KOIZUMI, N., MIZUGUCHI, H., SAKURAI, F., YAMAGUCHI, T., WATANABE, Y., and HAYAKAWA, T. (2003a). Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and α_v integrin-binding ablation. J. Virol. **77,** 13062–13072.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2003b). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. **5,** 267–276.

KRASNYKH, V., DOUGLAS, J., and VAN BEUSECHEM, W. (2000). Genetic targeting of adenoviral vectors. Mol. Ther. **1,** 391–405.

LEISSNER, P., LEGRAND, V., SCHLESINGER, Y., HADJI, D.A., VAN RAAIJ, M., CUSACK, S., PAVIRANI, A., and MEHTALI, M. (2001). Influence of adenoviral fiber mutations on viral encapsidation, infectivity and *in vivo* tropism. Gene Ther. **8,** 49–57.

LIEBER, A., HE, C., MEUSE, L., SCHOWALTER, D., KIRILLOVA, I., WINTHER, B., and KAY, M. (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. **71,** 8798–8807.

LIEVENS, J., SNOEYS, J., VEKEMANS, K., VAN LINTHOUT, S., DE ZANGER, R., COLLEN, D., WISSE, E., and DE GEEST, B. (2004). The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. Gene Ther. **11,** 1523–1531.

LIU, Q., ZAISS, A., COLARUSSO, P., PATEL, K., HALJAN, G., WICKHAM, T., and MURUVE, D. (2003). The role of capsid–endothelial interactions in the innate immune response to adenovirus vectors. Hum. Gene Ther. **14,** 627–643.

MAIZEL, J.V.J., WHITE, D.O., and SCHARFF, M.D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology **36,** 115–125.

MIYAZAWA, N., CRYSTAL, R., and LEOPOLD, P. (1999). Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. J. Virol. **75,** 1387–1400.

MIYAZAWA, N., LEOPOLD, P., HACKETT, N., FERRIS, B., WORGALL, S., FALCK-PEDERSEN, E., and CRYSTAL, R. (2001). Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. J. Virol. **73,** 6056–6065.

MIZUGUCHI, H., and HAYAKAWA, T. (2002). Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. Gene **285,** 69–77.

MIZUGUCHI, H., and HAYAKAWA, T. (2004). Targeted adenovirus vectors. Hum. Gene Ther. **15,** 1034–1044.

MIZUGUCHI, H., and KAY, M.A. (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum. Gene Ther. **9,** 2577–2583.

MIZUGUCHI, H., and KAY, M.A. (1999). A simple method for constructing E1 and E1/E4 deleted recombinant adenovirus vector. Hum. Gene Ther. **10,** 2013-2017.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., UTOGUCHI, N., WATANABE, Y., KAY, M.A., and HAYAKAWA, T. (2001). A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther. **8,** 730–735.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., ISHII-WATABE, A., UCHIDA, E., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2002). CAR- or α_v integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. Gene Ther. **9,** 769–776.

MORRAL, N., O'NEAL, W., RICE, K., LELAND, M., PIEDRA, P., AGUILAR-CORDOVA, E., CAREY, K., BEAUDET, A., and LANGSTON, C. (2002). Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. Hum. Gene Ther. **13,** 143–154.

MURUVE, D. (2004). The innate immune response to adenovirus vectors. Hum. Gene Ther. **15,** 1157–1166.

NAKAMURA, T., SATO, K., and HAMADA, H. (2003). Reduction of natural adenovirus tropism to the liver by both ablation of

fiber–coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. J. Virol. **77,** 2512–2521.

NICKLIN, S., WU, E., NEMEROW, G., and BAKER, A. (2005). The influence of adenovirus fiber structure and function on vector development for gene therapy. Mol. Ther. **12,** 384–393.

NISHIKAWA, M., TAKEMURA, S., TAKAKURA, Y., and HASHIDA, M. (1998). Targeted delivery of plasmid DNA to hepatocytes *in vivo*: Optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. J. Pharmacol. Exp. Ther. **287,** 408–415.

PASQUALINI, R., KOIVUNEN, E., and RUOSLAHTI, E. (1997). α_v integrins as receptors for tumor targeting by circulating ligands. Nat. Biotechnol. **15,** 542–546.

PHILPOTT, N., NOCIARI, M., ELKON, K., and FALCK-PEDERSEN, E. (2004). Adenovirus-induced maturation of dendritic cells through a PI3 kinase-mediated TNF-α induction pathway. Proc. Natl. Acad. Sci. U.S.A. **101,** 6200–6205.

REID, T., GALANIS, E., ABBRUZZESE, J., SZE, D., WEIN, L., ANDREWS, J., RANDLEV, B., HEISE, C., UPRICHARD, M., HATFIELD, M., ROME, L., RUBIN, J., and KIRN, D. (2002). Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): Phase II viral, immunologic, and clinical endpoints. Cancer Res. **62,** 6070–6079.

SAKURAI, F., MIZUGUCHI, H., YAMAGUCHI, T., and HAYAKAWA, T. (2003). Characterization of *in vitro* and *in vivo* gene transfer properties of adenovirus serotype 35 vector. Mol. Ther. **8,** 813–821.

SCHIEDNER, G., BLOCH, W., HERTEL, S., JOHNSTON, M., MOLOJAVYI, A., DRIES, V., VARGA, G., VAN ROOIJEN, N., and KOCHANEK, S. (2003). A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. Hum. Gene Ther. **14,** 1631–1641.

SCHNELL, M., ZHANG, Y., TAZELAAR, J., GAO, G., YU, Q., QIAN, R., CHEN, S., VARNAVSKI, A., LECLAIR, C., RAPER, S., and WILSON, J. (2001). Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol. Ther. **3,** 708–722.

SHAYAKHMETOV, D., GAGGAR, A., NI, S., LI, Z., and LIEBER, A. (2005). Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. J. Virol. **72,** 7478–7491.

SMITH, T., IDAMAKANTI, N., KYLEFJORD, H., ROLLENCE, M., KING, L., KALOSS, M., KALEKO, M., and STEVENSON, S.C. (2002). *In vivo* hepatic adenoviral gene delivery occurs independently of the coxsackievirus–adenovirus receptor. Mol. Ther. **5,** 770–779.

SMITH, T., IDAMAKANTI, N., ROLLENCE, M.L., MARSHALL-NEFF, J., KIM, J., MULGREW, K., NEMEROW, G.R., KALEKO, M., and STEVENSON, S.C. (2003a). Adenovirus serotype 5 fiber shaft influences *in vivo* gene transfer in mice. Hum. Gene Ther. **14,** 777–787.

SMITH, T., IDAMAKANTI, N., MARSHALL-NEFF, J., ROLLENCE, M., WRIGHT, P., KALOSS, M., KING, L., MECH, C., DINGES, L., IVERSON, W., SHERER, A., MARKOVITS, J., LYONS, R., KALEKO, M., and STEVENSON, S. (2003b). Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. Hum. Gene Ther. **14,** 1595–1604.

TOMKO, R.P., XU, R., and PHILIPSON, L. (1997). HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. U.S.A. **94,** 3352–3356.

VIGNE, E., DEDIEU, J., BRIE, A., GILLARDEAUX, A., BRIOT, D., BENIHOUD, K., LATTA-MAHIEU, M., SAULNIER, P., PERRICAUDET, M., and YEH, P. (2003). Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation. Gene Ther. **10,** 153–162.

WICKHAM, T.J. (2000). Targeting adenovirus. Gene Ther. **7,** 110–114.

WICKHAM, T.J., MATHIAS, P., CHERESH, D.A., and NEMEROW, G.R. (1993). Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. Cell **73,** 309–319.

WICKHAM, T.J., FILARDO, E.J., CHERESH, D.A., and NEMEROW, G.R. (1994). Integrin $\alpha_v\beta_5$ selectively promotes adenovirus mediated cell membrane permeabilization. J. Cell Biol. **127,** 257–264.

XU, Z.-L., MIZUGUCHI, H., ISHII-WATABE, A., UCHIDA, E., MAYUMI, T., and HAYAKAWA, T. (2001). Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene **272,** 149–156.

ZINN, K., SZALAI, A., STARGEL, A., KRASNYKH, V., and CHAUDHURI, T. (2004). Bioluminescence imaging reveals a significant role for complement in liver transduction following intravenous delivery of adenovirus. Gene Ther. **11,** 1482–1486.

ZSENGELLER, Z., OTAKE, K., HOSSAIN, S.A., BERCLAZ, P.Y., and TRAPNELL, B.C. (2000). Internalization of adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. J. Virol. **74,** 9655–9667.

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Design and synthesis of a Tat-related gene transporter: A tool for carrying the adenovirus vector into cells

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Abstract—A Tat-related peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide, designed to transport an Adenovirus vector (Ad) into cells, was synthesized. The synthetic peptide was conjugated to Ad, which potentially can act as an efficient carrier of heterologous genes into cells. The Tat-related peptide was synthesized using the solid phase method and then was coupled to the heterofunctional cross-linking reagent, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester. The resulting peptide-succinimidohexanoic acid *N*-hydroxysuccinimide ester was conjugated to Ad containing the luciferase gene. B16BL6 cells infected with the peptide-conjugated Ad luciferase gene construct exhibit a 50-fold greater luciferase activity than B16BL6 cells infected with wild-type Ad containing the luciferase gene.

Gene therapy has attracted much attention as a potential clinical treatment/cure for intractable diseases.[1] A key to the successful implementation of gene therapy protocols is the design of the transgenesis vector. Adenovirus vectors (Ad) are often used as transport agents during gene therapy experiments and trials since they exhibit suitable transduction and gene-expression properties; but for routine clinical procedures, more efficient transfer vectors need to be developed. Previously, we showed that an Arg-Gly-Asp(RGD)-related peptide that binds to integrin functions as an efficient auxiliary transporter of Ad.[2] The RGD-related peptide, when covalently bound to Ad, transports Ad into dendritic cells via interaction with integrins. For this report, a different type of Ad auxiliary transporter was designed, synthesized, and shown to greatly increase the amount of Ad (containing the luciferase gene) transferred into cells.

The human immunodeficiency virus (HIV)-1 protein, Tat, is a transcriptional activator of HIV and can cross both the plasma and nuclear membranes. Tat contains 86 amino acids, but its translocation activity is associated with the peptide sequence, Tat(48–60), (GlyArgLysLysArgArgGlnArgArgArgProProGln: GRKKRRQRRRPPQ).[3] Futaki et al. reported that certain synthetic arginine-rich peptides can readily cross cell membranes and that the optimal number of arginines required for efficient translocation is approximately eight.[4] We designed the peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide (Ac-GRRRRRQRRRPPQGC-NH_2), to be an efficient auxiliary transporter of Ad. Since Futaki et al.[4] reported that the number of arginine residues correlates with translocation ability, the sequence, Ac-GRRRRRQRRRPPQGC-NH_2, was designed so that the two lysines found in Tat(48–60) were replaced with arginines. A C-terminal cysteine was added so that the peptide could be linked to Ad through the heterofunctional cross-linking reagent 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS),[5] which reacts with amine and sulfhydryl moieties (Fig. 1). The peptide was synthesized using an Applied Biosystems Peptide Synthesizer 433A-1. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids [Fmoc-Gly-OH; Fmoc-Pro-OH; N^{α}-Fmoc-N^{G}-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony-

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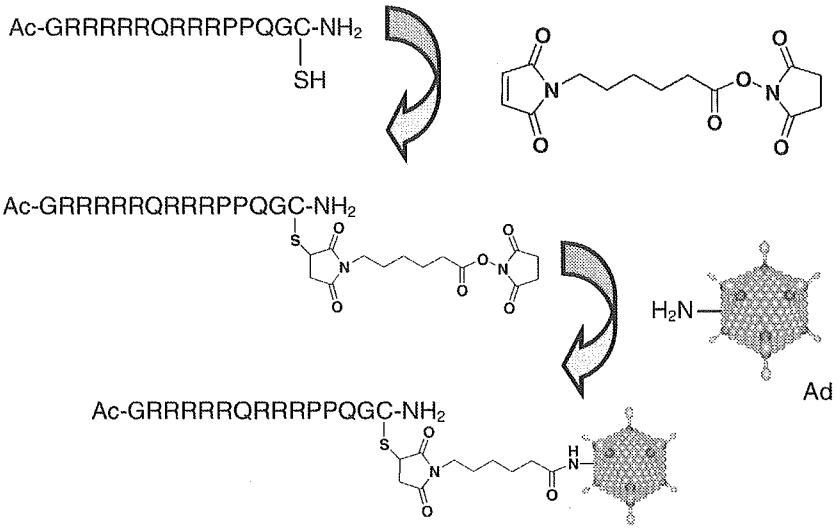

Figure 1. Synthesis of a peptide–Ad conjugate that acts as an efficient heterologous gene transporter.

larginine: Fmoc-Arg(Pbf)-OH; N^{α}-Fmoc-*S*-tritylcysteine: Fmoc-Cys(Trt)-OH; and Fmoc-Gln(Trt)-OH] were coupled in a stepwise manner to Rink amide resin[6] (PE Biosystems. Amino content: 0.67 mequiv/g, 379 mg, 0.25 mmol) using the coupling reagent, 2-(1-*H*-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[7] in *N*-methylpyrrolidone (NMP). After each coupling step, the Fmoc group was removed using 20% piperidine/NMP. The synthetic Fmoc-Gly-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Pro-Pro-Gln(Trt)-Gly-Cys(Trt)-Rink amide resin was treated with 20% piperidine/NMP and then treated with acetic anhydride. The peptide was cleaved from the resin with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (95:2.5:2.5). The resulting crude peptide, (Ac-GRRRRRQRRRPPQGC-NH_2, 410 mg yield), was purified using RP-HPLC.[8] The HPLC profile of the crude peptide mixture is shown in Figure 2 The yield of the purified peptide was 202 mg (28% as calculated from the amino content of the used resin).

The purified peptide (40 mg, 14 μmol), dissolved in PBS (pH 7.2, 500 μL), and the heterofunctional cross-linkage reagent (MHS: 4.3 mg, 14 μmol), dissolved in dimethylsulfoxide (DMSO, 10 μL), were combined and then stirred for 0.5 h. We attempted to purify the product, Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 (SHS: 6-succinimidohexanoic acid *N*-hydroxysuccinimide ester), using HPLC, but could not—the *N*-hydroxysuccinimide ester hydrolyzes easily in water; therefore, the reaction product mixture was frozen immediately and kept at −80 °C until needed. While gently stirring, Ad-Luc, whose chromosome encodes the heterologous luciferase gene, was reacted with Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 at 37 °C for 45 min. To test the relative transduction efficiency of the peptide–Ad conjugate [Tatpep-(Ad-Luc)],

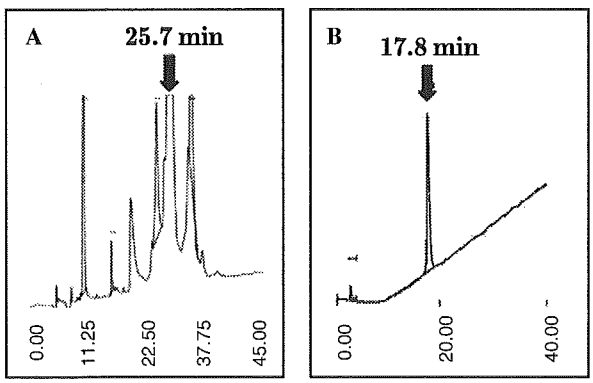

Figure 2. HPLC profile of synthetic crude Ac-GRRRRRQ-RRRPPQGC-NH_2. (A) Preparative HPLC of crude synthetic peptide. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10–70% CH_3CN over the course of 60 min. The absorbance was measured at 220 nm. (B) Analytical HPLC of purified sample. Column: Inertsil ODS-3 (4.6 × 250 mm). Flow rate: 1 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 5–20% CH_3CN over the course of 40 min. The absorbance was measured at 220 nm.

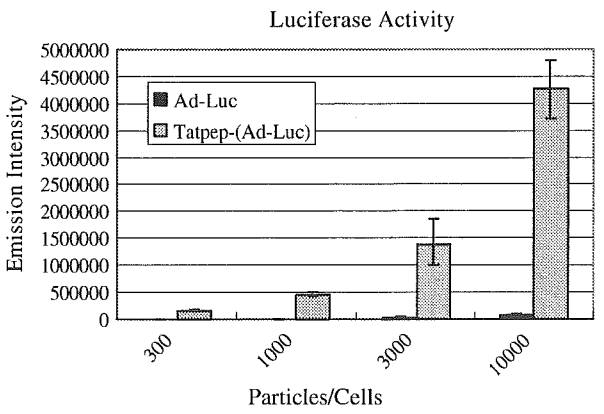

Figure 3. Transduction efficiency of Ad-Luc and Tatpep-(Ad-Luc) into B16BL6 cells Cells (2×10^4) were incubated with 300, 1000, 3000 or 10,000 particles/cell of Ad-Luc (solid bars) or Tatpep-(Ad-Luc) (gray bars). Luciferase activity, which was determined using a Luciferase Assay System Kit (Promega, USA) and a Microlumat Plus LB96 (Perkin-Elmer, USA) after lysing the cells with Luciferase Cell Culture Lysis Reagent (Promega, USA), was measured at the end of a 24 h incubation. The bars report the mean relative unit of light per well ± SD ($n = 3$).

B16BL6 cells were incubated with it or with Ad-Luc for 24 h, at which time luciferase activity was measured (Fig. 3). B16BL6 cells were used because the Coxackie-adenovirus receptor, which transports Ad across the plasma membrane, is nearly absent.[9] At concentrations of 300 and 1000 particles/cell, cells that were exposed to Ad-Luc did not glow, while those exposed to Tatpep-(Ad-Luc) construct clearly did. Ad-Luc infected cells glowed weakly at doses of 3000 and 10,000 particles/cell, while Tatpep-(Ad-Luc) exhibited strong luciferase activity at the same concentrations. The transduction activity of Tatpep-(Ad-Luc) is about 50-fold greater than that of Ad-Luc—a remarkable finding.

In summary, we designed the peptide, Ac-GRRRRRQ-RRRPPQGC-NH_2, to be an efficient auxiliary transporter of Ad into cells. Ad, when covalently bound to this synthetic peptide, exhibits a transduction ability 50-fold greater than does Ad alone. This modified Ad is a promising experimental tool for transduction studies.

Acknowledgment

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References and notes

1. (a) Crystal, R. G. *Science* **1995**, *270*, 404; (b) Wilson, J. M. *N. Engl. J. Med.* **1996**, *334*, 1185.
2. Maeda, M.; Kida, S.; Hojo, K.; Eto, Y.; Gao, J.-Q.; Kurachi, S.; Sekiguchi, F.; Mizuguchi, H.; Hayakawa, T.; Mayumi, T.; Nakagawa, S.; Kawasaki, K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 621.
3. (a) Fawell, S.; Seery, J.; Daikh, Y.; Moore, C.; Chen, L. L.; Pepinsky, B.; Barsoum, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 664; (b) Vives, E.; Brodin, P.; Lebleu, B. *J. Biol. Chem.* **1997**, *272*, 16010.
4. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836.
5. (a) Hashida, S.; Imagawa, M.; Inoue, S.; Ruan, K. H.; Ishikawa, E. *J. Appl. Biochem.* **1984**, *6*, 56; (b) Fargeas, C.; Hommel, M.; Maingon, R.; Dourado, C.; Monsigny, M.; Mayer, R. *J. Clin. Microbiol.* **1996**, *34*, 241.
6. Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787.
7. Knorr, R.; Trzecial, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927.
8. $[\alpha]_D^{20}$ −68.8° (*c* 1.0, H_2O). Amino acid ratios in an acid hydrolysate Glu 2.20; Gly 2.00; Arg 8.35; Pro 2.06 (average recovery 87%). TOF-MS *m/z*: 1975.9 (calcd for $C_{77}H_{142}N_{42}O_{18}S$ 1975.1).
9. Okada, N.; Tsukada, Y.; Nakagawa, S.; Mizuguchi, H.; Mori, K.; Saito, T.; Fujita, T.; Yamamoto, A.; Hayakawa, T.; Mayumi, T. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 173.

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Efficient regulation of gene expression using self-contained fiber-modified adenovirus vectors containing the tet-off system

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Abstract

Previously, we developed single adenovirus (Ad) vectors that contained the gene of interest in the E1 deletion region and the transactivator gene for the tetracycline-controllable expression system in the E3 deletion region. In the present study, we improved the Ad vector-mediated tetracycline-controllable expression system by the fiber modification of Ad. We developed fiber-modified Ad vectors containing the tet-off system, which are effective in overcoming the limitations of conventional Ad vectors, specifically their inefficient gene transfer into cells lacking the primary receptor, the coxsackievirus and adenovirus receptor (CAR). Ad vectors containing the tet-off system with an Arg–Gly–Asp (RGD) peptide in the HI loop of the fiber knob or the Ad type 35 fiber greatly improved transduction efficiency (more than 1–2-log orders) into the cells lacking CAR expression but expressing αv integrin or CD46, respectively. They exhibited vastly higher regulation of gene expression by doxycycline. The combination of fiber-modified Ad vectors and the tetracycline-controllable expression system should offer a powerful tool for gene therapy and gene transfer experiment.

Keywords: Gene therapy; Adenovirus vector; Fiber; Tetracycline; Gene regulation

1. Introduction

Regulated transgene expression systems provide a valuable tool for both studies of gene function and safe, effective gene therapy. The tetracycline-controllable expression system has been most widely used, because it has several advantages over other regulated

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gene expression systems; e.g., tight on/off regulation, high inducibility, fast response times, lack of a pleiotropic effect due to the use of tetracycline operons derived from bacteria, and a well-characterized inducer (tetracycline), etc. [1,2]. The tet-off system [1], which uses the tetracycline-responsive transcriptional activator (tTA), and the tet-on system [2], which uses the reverse tetracycline-responsive transcriptional activator (rtTA), provide negative and positive control of transgene expression, respectively.

Previously, we developed single adenovirus (Ad) vectors containing the gene of interest in the E1 deletion region and *tTA* or *rtTA* gene in the E3 deletion region, and showed that Ad vectors containing the tet-off system mediated high regulation of gene expression by doxycycline [3]. However, Ad vectors containing the tet-on system exhibit a much lower level of regulation (less than 10–30-fold of regulation factor (ratio of maximum transgene expression to minimum transgene expression)) of transgene expression than Ad vectors containing the tet-off system [3]. We therefore developed Ad vectors that overcame this lower level of regulation in the tet-on system by introducing the latest generation rtTA and tetracycline-controlled transcriptional silencer [4].

In the present study, in order to further improve the self-contained Ad vectors having a tetracycline-controllable expression system, fiber-modified Ad vectors were employed to deliver the transgene cassette. Ad type 5 along with Ad type 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. However, Ad vector-mediated transduction is quite low when the cells do not express sufficient levels of the primary receptor, called the coxsackievirus and adenovirus receptor (CAR) [5,6]. Modification of fiber protein is an attractive strategy for overcoming the limitations imposed by the CAR-dependence of Ad infection [7]. We and other groups have shown that Ad vectors containing the RGD motif in the HI loop of the fiber knob and Ad vectors containing the Ad type 35 fiber greatly increase the efficiency of transduction to a variety of CAR-deficient cells [7–16]. αv integrin [8] and CD46 (membrane cofactor protein) [17,18] could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by fiber-modified Ad vectors containing the RGD peptides or Ad type 35 fiber, respectively [7]. We here show the successful generation of self-contained Ad vectors having both fiber-modification and the tet-off system. We report the application and characteristics of gene transfer and regulation of fiber-modified Ad vectors containing the tet-off system.

2. Materials and methods

2.1. Cells

SK HEP-1 (endothelial cell line from human liver) [19], LNZ308 (human glioblastoma multiforme; kindly provided by Dr. M. Tada, Hokkaido University, Hokkaido, Japan) [20], and 293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). NIH3T3 cells (mouse embryo fibroblast; Human Science Research Resources Bank, Japan, JCRB0615) were cultured with minimum essential medium (MEM) supplemented with 10% FCS. CHO (Chinese hamster ovary) cells were cultured with MEM alpha medium supplemented with 10% FCS. CHO–CD46 cells were stable transformants generated by the transfection of pcDNA3.1-CD46 (described below) into CHO cells and selection with hygromycin (GIBCO-BRE, Rockville, MD). CHO–CD46 cells stably expressed the BC2 isoform of human CD46 (Fig. 3B).

2.2. Plasmid and virus

Vector plasmid pAdHM49 was constructed as described below. First, pHM15-ITR10 was constructed by the ligation of NdeI/MunI-digested pHM15-ITR16 [4] and NdeI/MunI-digested pEco-ITR10, a derivative of pEco-ITR9 [13]. pHM15-ITR10 contains the right end of the Ad genome (bp 27332-right end with deletion of bp 27865 to bp 30995) with a Csp45I site in the E3 deletion region, an XbaI site between the E4 region and 3′ inverted terminal repeat (ITR), and an Ad type 35 fiber (Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail)-coding sequence. Then, Csp45I/XbaI-digested pAdHM19 was ligated with Csp45I/NdeI-digested pHM15-ITR10, resulting in pAdHM49-1. Finally, pAdHM49 was constructed by changing the ClaI site at the right end of the Ad genome of pAdHM49-1 into a PacI site using

oligonucleotides (5′-CGTTAATTAA-3′) (PacI recognition sequences are underlined). pAdHM49 has a complete E1/E3-deleted Ad genome with an I-CeuI, a SwaI, and a PI-SceI site in the E1 deletion region, a Csp45I site in the E3 deletion region, an XbaI site between the E4 region and 3′ITR, PacI sites at both ends of the Ad genome, and the chimeric fiber-coding sequence of the Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail. pAdHM51, which contains an I-CeuI, a SwaI, and a PI-SceI site in the E1 deletion region, a ClaI site in the E3 deletion region, an XbaI site between the E4 region and 3′ITR, PacI sites at both ends of the Ad genome, and a Csp45I site between positions 32679 and 32680 of the Ad genome (residues threonine-546 and proline-547 of the fiber protein), was similarly constructed (Fig. 1). pAdHM51-RGD was constructed by insertion of oligonucleotide 1 (5′-CGGCCTGTGACTGCCGCGGAGACTGTTTCTGCGATG-3′) and oligonucleotide 2 (5′-CGCATCGCAGAAACAGTCTCCGCGGCAGTCACAGGC-3′), which correspond to the RGD (RGD-4C) peptide (CDCRGDCFC) with high affinities to integrins (αvβ3 and αvβ5) [21], into a Csp45I site of pAdHM51.

pHM13-CMVi-tTA was constructed by cloning the cytomegalovirus (CMV) promoter with intron A (derived from pGeneGrip (Gene Therapy Systems, San Diego, CA)), *tTA* gene (derived from pTet-Off (Clontech, Palo Alto, CA)), and SV40 late P(A) signal sequences into pHM13 [22]. pAdHM20-tTA1, pAdHM49-tTA1, and pAdHM51-RGD-tTA1 were constructed by the ligation of ClaI-digested pHM13-CMVi-tTA into ClaI-digested pAdHM20 [22], Csp45I-digested pAdHM49, and ClaI-digested pAdHM51-RGD, respectively. pHM5-TRE-SEAP and pHM5-TRE2-SEAP are shuttle plasmids for insertion of the gene of interest into the E1 deletion region of the vector plasmid. pHM5-TRE-SEAP contains the sequence of the tet-responsive promoter (derived from pTRE (Clontech)), the secreted alkaline phosphatase (SEAP) gene (derived from pSEAP2-Control (Clontech)), and the bovine growth hormone (BGH) poly (A) signal. pHM5-TRE2-SEAP contains the tight tet-responsive promoter (derived from pTRE-Tight (Clontech)) instead of the tet-responsive promoter in pHM5-TRE-SEAP. pAdHM20-tTA1-SEAP4 was constructed by the ligation of I-CeuI/PI-SceI-digested pHM5-TRE-SEAP with I-CeuI/PI-SceI-digested pAdHM20-tTA1. pAdHM20-tTA1-SEAP6, pAdHM49-tTA1-SEAP6, and pAdHM51-RGD-tTA1-SEAP6 were constructed by the ligation of I-CeuI/PI-SceI-digested pHM5-TRE2-SEAP with I-CeuI/PI-SceI-digested pAdHM20-tTA1, pAdHM49-tTA1, and pAdHM51-RGD-tTA1, respectively.

To generate the virus, pAdHM20-tTA1-SEAP4, pAdHM20-tTA1-SEAP6, pAdHM49-tTA1-SEAP6,

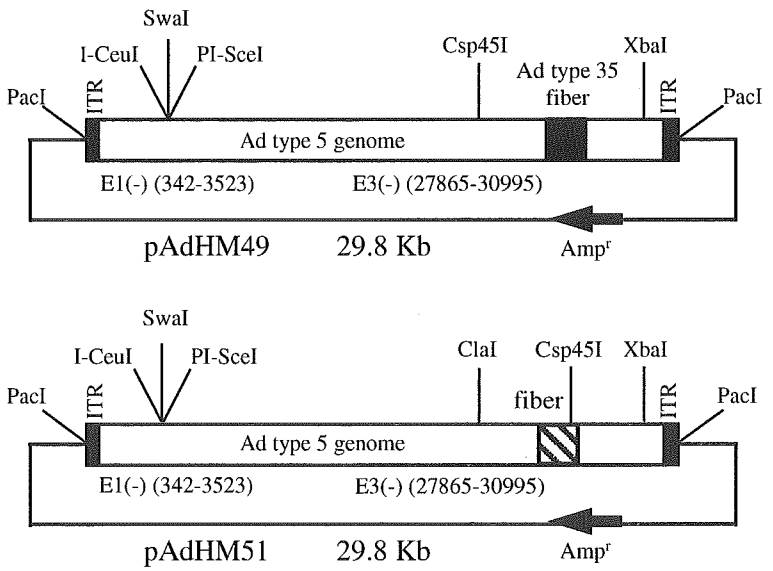

Fig. 1. The structure of the vector plasmids pAdHM49 and -51.

and pAdHM51-RGD-tTA1-SEAP6 were digested with PacI and purified by phenol–chloroform extraction and ethanol precipitation. Linearized DNAs were transfected into 293 cells plated in a 60 mm dish with SuperFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Viruses (AdOff-SEAP4, AdOff-SEAP6, AdF35-Off-SEAP6, AdRGD-Off-SEAP6) were prepared as described previously [23]. Viruses were purified with CsCl gradient centrifugation, dialyzed with the solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −70 °C. Ad-SEAP2, which contains the CMV promoter/enhancer-driven SEAP expression cassette in the E1 deletion region, was previously prepared [4]. Determination of virus particle titers was accomplished spectrophotometrically by the methods of Maizel et al. [24]. The Ad vectors used in the present study are summarized in Table 1.

pcDNA3.1-CD46 was constructed by insertion of the gene coding the human CD46 BC2 isoform, which was generated by PCR using primers (forward, 5′-atggagcctcccggccgccgcgagtgtccc-3′; reverse, 5′-cgcggccgcctattcagcctctctgctctgctg-3′), into pcDNA3.1-Hyg (Invitrogene). Sequencing of the gene coding the human CD46 BC2 isoform in pcDNA3.1-CD46 verified that the clone contained the appropriate sequences.

2.3. Adenovirus-mediated gene transduction in vitro

The cells (1×10^4 cells) were seeded into a 96-well dish. On the following day, they were transduced with the Ad vector (500 vector particles (VP)/cell) for 1.5 h. The cells transduced with the Ad vector containing the tet-off system were cultured with medium containing 10 ng/ml of doxycycline (Clontech). Tet-system-proved FCS (Clontech), a tetracycline-free serum determined to be optimal for the tetracycline-controllable expression system, was used as FCS. Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, the SEAP level in the medium was measured using a Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech).

2.4. Flow cytometric analysis

CHO, CHO–CD46, SK HEP-1, and LNZ308 cells were suspended in staining buffer (phosphate buffered saline containing 1% bovine serum albumin) containing fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD46 antibody (Pharmingen, San Diego, CA, USA). After washing with the sorting solution, the stained cells (10^4 cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson).

3. Results and discussion

3.1. Newer vector plasmids

In order to combine fiber-modified Ad vectors and the tetracycline-controllable expression system, newer Ad vector systems were first developed (Fig. 1). The vector plasmid pAdHM49 contains the chimeric fiber proteins (Ad type 35 fiber knob, Ad type 35 fiber shaft, and Ad type 5 fiber tail), and an I-CeuI/SwaI/PI-SceI site, a Csp45I site, and an XbaI site in the E1 deletion region, the E3 deletion region and the region between the E4 region and 3′ITR, respectively. The vector plasmid pAdHM51 contains a unique Csp45I site in the HI loop-coding sequence of the fiber knob as well as an I-CeuI/SwaI/PI-SceI site in the E1 deletion region, a ClaI site in the E3 deletion region, and an XbaI site between the E4 region and 3′ITR. Any foreign ligands can be easily displayed in the HI loop of the fiber knob of Ad vectors by cloning its gene into their regions using simple in vitro ligation as

Table 1
Adenovirus vectors used in the present study

Name	Fiber type	E1 deletion region	E3 deletion region
Ad-SEAP2	Type 5 fiber	CMV promoter+SEAP	
AdOff-SEAP4	Type 5 fiber	TRE promoter+SEAP	CMV promoter+tTA
AdOff-SEAP6	Type 5 fiber	TRE-Tight promoter+SEAP	CMV promoter+tTA
AdRGD-Off-SEAP6	RGD peptide in the HI-loop of the fiber knob	TRE-Tight promoter+SEAP	CMV promoter+tTA
AdF35-Off-SEAP6	Chimeric type 5 fiber tail and type 35 fiber knob and shaft	TRE-Tight promoter+SEAP	CMV promoter+tTA

previously described [12,14]. In both vector plasmids, the gene of interest can also be introduced into the E1 deletion region as well as the E3 deletion region and the region between the E4 region and 3′ITR by simple in vitro ligation using a series of shuttle plasmids developed previously [4,22]. In the present study, the coding sequence of the RGD peptide, which binds with high affinities to both αvβ3 and αvβ5 [21], was introduced into the HI loop-coding region of the fiber knob (a Csp45I site) of pAdHM51.

3.2. Effect of the tet-responsive element on the regulated gene expression

Previously, we developed self-contained Ad vectors having a tetracycline-controllable expression system [3,22]. These vectors contain the original tet-responsive promoter, which is a hybrid of the tet-responsive element (TRE) containing a seven tet-operator (tetO) sequence and the minimal CMV promoters. Recently, a modified tet-responsive promoter derived from pTRE-Tight (Clontech) was developed. Both promoters contain a seven tetO sequence. The sequences among the tetO sequence and the minimal CMV promoter differ between the original and the modified tet-responsive promoters. The modified tet-responsive promoter contains a deleted minimal CMV promoter.

First, we performed a functional comparison of these two promoters cloned in the Ad vectors (Fig. 2). AdOff-SEAP4 contains the original tet-responsive promoter-driven SEAP expression cassette in the E1 deletion region as well as the tTA expression cassette in the E3 deletion region. AdOff-SEAP6 contains a modified tet-responsive promoter instead of the original tet-responsive promoter in AdOff-SEAP4. SK HEP-1 and HeLa cells, both of which express sufficient levels of CAR, were transduced with AdOff-SEAP4 or AdOff-SEAP6 at 500 VP/cell, and the basal and induced SEAP production in the medium, in which the cells were cultured without doxycycline or with 10 ng/ml of doxycycline, respectively, were measured. Both cell types transduced with AdOff-SEAP6 showed lower basal SEAP production than those transduced with AdOff-SEAP4, although the induced SEAP production in the cells transduced with AdOff-SEAP6 was also lower than that with AdOff-SEAP4. Importantly, the regulation factor[s]

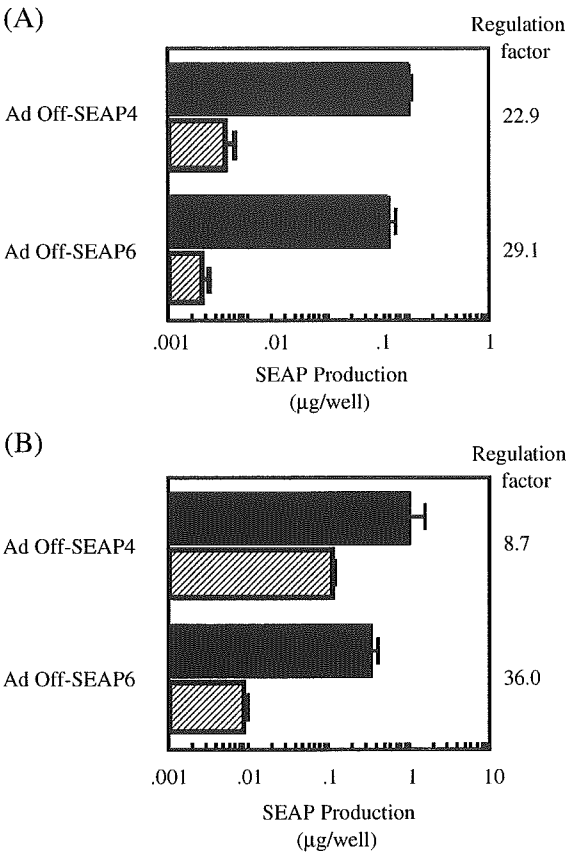

Fig. 2. Effect of the tet-responsive element on the regulated SEAP production. SK HEP-1 (A) and HeLa (B) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP4 or AdOff-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the two cell types were as follows: SK HEP-1, 0.0006; HeLa, 0.0009 (μg/well).

(ratio of maximum SEAP production to basal SEAP production) by AdOff-SEAP6 were higher than those by AdOff-SEAP4; the regulation factor by AdOff-SEAP6 were 29.1 (SK HEP-1 cells) and 36.0 (HeLa cells), while those by AdOff-SEAP4 were 22.9 (SK HEP-1 cells) and 8.7 (HeLa cells). These results suggest that the single Ad vector containing the modified-tet responsive promoter and the tet-off system showed lower basal transgene expression and higher regulation of transgene expression by doxycycline,

while the single Ad vector containing the original tet-responsive promoter showed higher induced (maximum) transgene expression. The modified tet-responsive promoter was used in the following experiments.

3.3. Fiber-modified Ad vectors containing the tet-off system

Fiber-modified Ad vectors containing the tet-off system were developed to transduce various types of cells, including CAR-deficient cells. By using pAdHM51-RGD and pAdHM49, Ad vectors containing both the tet-off system and the fiber modification, such as the addition of the RGD peptide in the HI loop of the fiber knob or substitution of Ad type 5 fiber for Ad type 35 fiber, were easily generated. The resulting Ad vectors, AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, were functionally compared with AdOff-SEAP6. CHO and CHO–CD46 cells were used (Fig. 3). Neither of these cells expresses CAR, but both express αv integrin. CHO cells are CD46 negative, while CHO–CD46 cells are CD46 positive (Fig. 3A,B). AdRGD-Off-SEAP6 achieved approximately 46-times higher SEAP production in CHO cells than AdOff-SEAP6 in the absence of doxycycline (Fig. 4). In contrast, in the case of CHO–CD46 cells, AdF35-Off-SEAP6 achieved approximately 40-times higher SEAP production than AdOff-SEAP6 in the absence of doxycycline (Fig. 4B). In both cases, fiber-modified Ad vectors showed higher regulation of SEAP production by doxycycline. AdOff-SEAP6 showed very low regulation of SEAP production by doxycycline, probably due to the lower transduction efficiency.

In order to further examine the functionality of AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, SEAP expression profiles were examined in SK HEP-1, LNZ308, and NIH3T3 cells (Fig. 5). SK HEP-1 cells express sufficient levels of CAR, αv integrin [25], and CD46 (Fig. 3C). LNZ308 cells express αv integrin and CD46 (Fig. 3D), but not CAR [25]. NIH3T3 cells

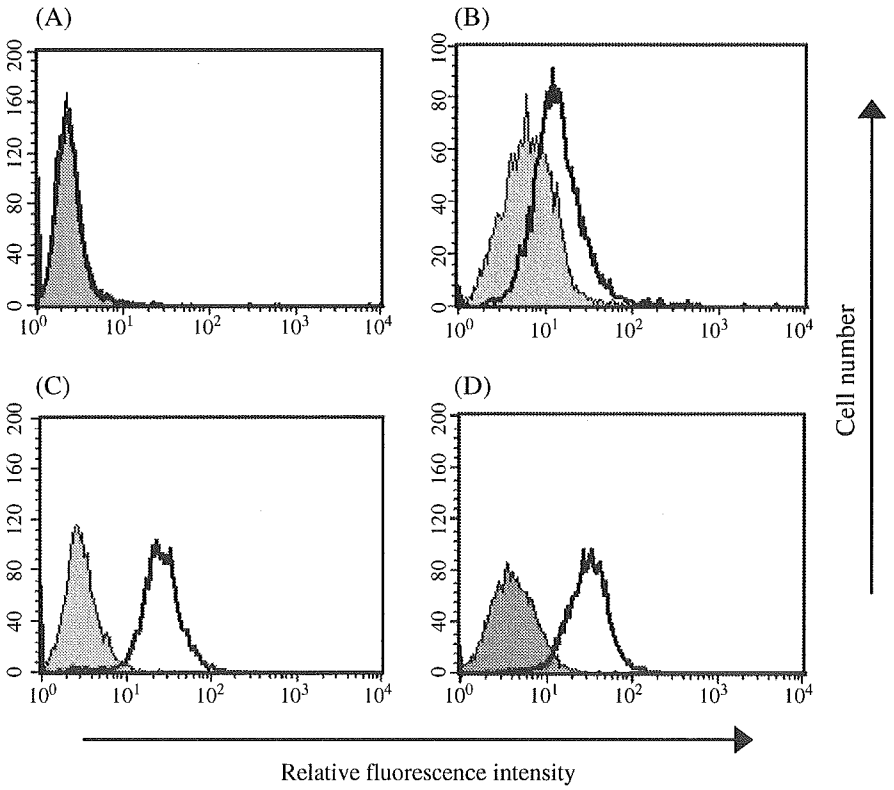

Fig. 3. CD46 expression in CHO, CHO–CD46, SK HEP-1, and LNZ308 cells. CHO (A), CHO–CD46 (B), SK HEP-1 (C), and LNZ308 (D) cells were stained with FITC-conjugated anti-CD46 antibodies, and subsequently analyzed by a flowcytometer.

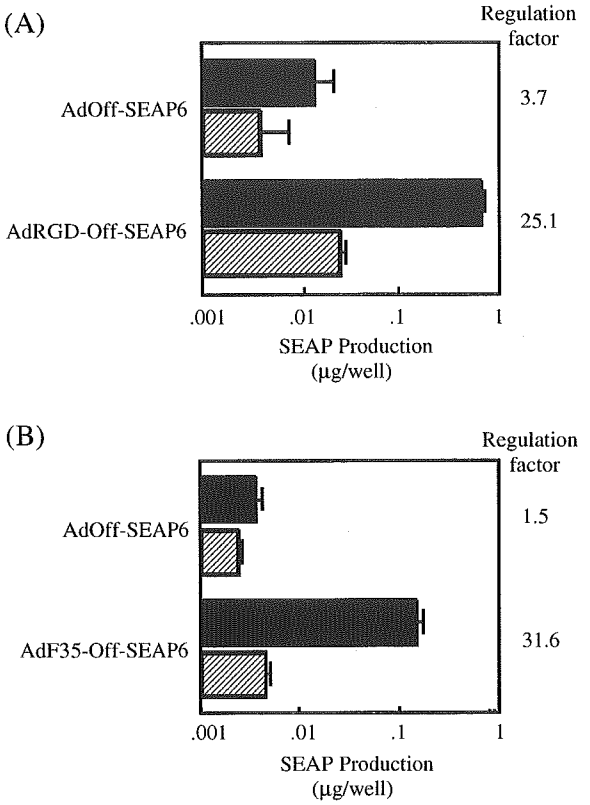

Fig. 4. Fiber-modified Ad vector-mediated tet-off systems in CHO and CHO–CD46 cells. CHO (A) and CHO–CD46 (B) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGD-Off-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the two cell types were as follows: CHO, 0.0008; CHO–CD46, 0.0002 (μg/well).

express αv integrin, but not CAR [25] or CD46. In SK HEP-1 cells, all the vectors, AdOff-SEAP6, AdRGD-Off-SEAP6 and AdF35-Off-SEAP6, showed high SEAP production in the absence of doxycycline as well as high regulation factors by doxycycline (Fig. 5A). In contrast, only AdRGD-Off-SEAP6 and AdF35-Off-SEAP6 achieved high regulation factor by doxycycline and high SEAP production in the absence of doxycycline in LNZ308 cells (Fig. 5B), while only AdRGD-Off-SEAP6 achieved high regulation factor and high SEAP production in NIH3T3 cells (Fig. 5C). AdOff-SEAP6 showed lower SEAP production in LNZ308 and NIH3T3 cells (Fig. 5B, C). Taken together, these results indicate that fiber-modified Ad vectors containing the tet-off system overcame the

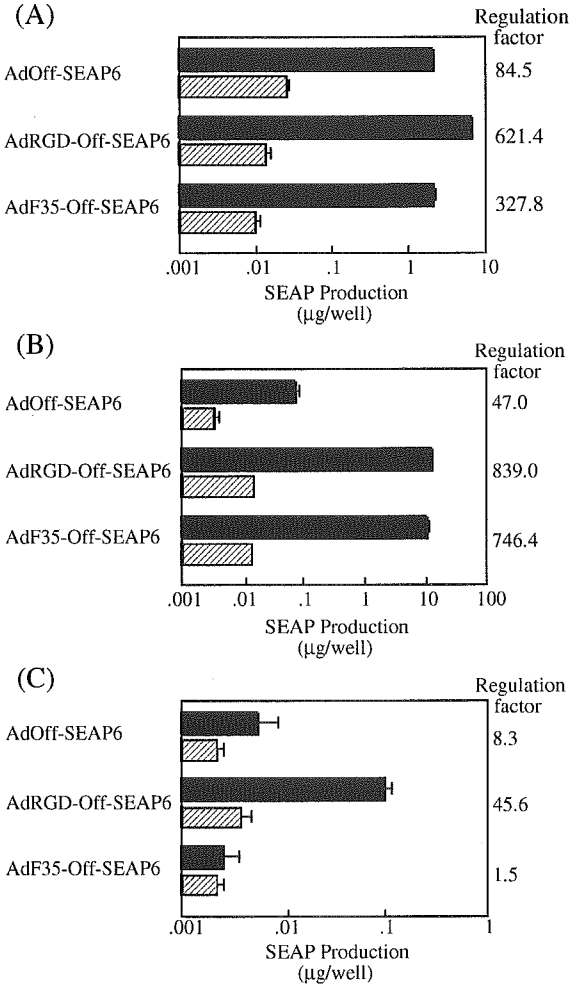

Fig. 5. Fiber-modified Ad vector-mediated tet-off systems in various types of cells. SK HEP-1 (A), LNZ308 (B), and NIH3T3 (C) cells, seeded into a 96-well dish, were transduced with AdOff-SEAP6, AdRGDOff-SEAP6, or AdF35Off-SEAP6 at 500 VP/cell, and cultured without (closed columns) or with doxycycline (slashed columns) (10 ng/ml). Thirty-six hours later, the medium was changed and the cells were cultured for an additional 36 h. Then, SEAP production in the medium was determined. The regulation factor is the ratio of induced SEAP production to uninduced SEAP production. The data are expressed as the mean ± S.D. ($n=3$). The mean background values of SEAP production in the three cell types were as follows: SK HEP-1, 0.0023; LNZ308, 0.0008; NIH3T3, 0.0004 (μg/well).