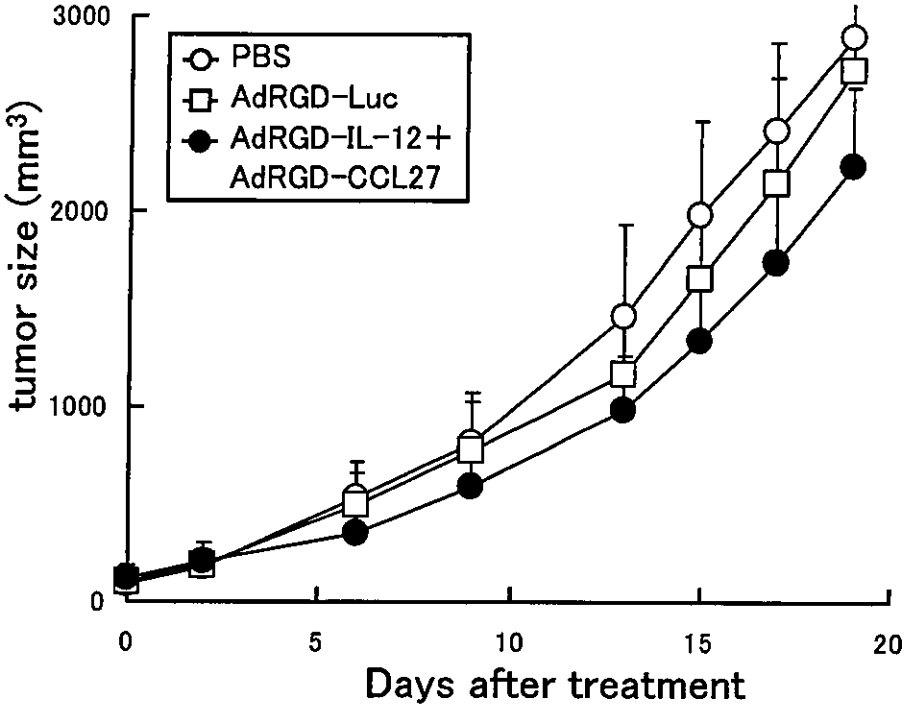

Fig. 3 Anti-tumor effect induced by the combination of AdRGD-IL-12 and AdRGD-CCL27 is T cell-dependent

Balb/c nude mice were inoculated intradermally with OV-HM cells (1×10^6 cells/mouse). After one week, 50 μl of PBS, 2×10^7 PFU of AdRGD-Luc, or AdRGD-IL-12 plus AdRGD-CCL27, in total of 2×10^7 PFU at the ratio of 9:1, were intratumorally injected . Tumor size was measured twice a week.

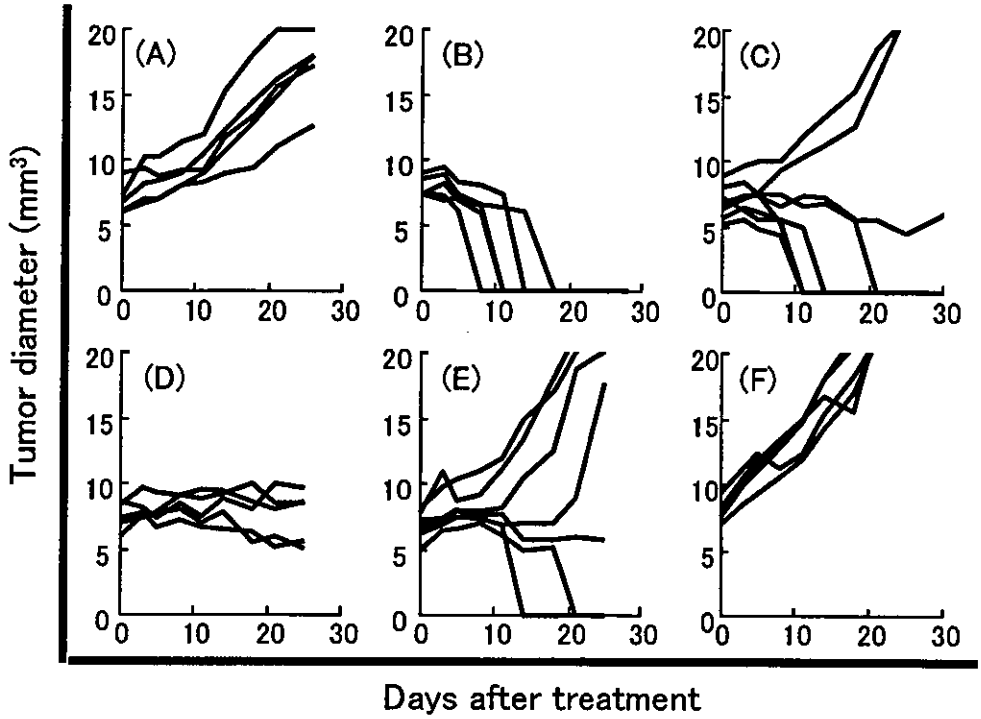

Fig. 4 Both CD4 or CD8 T cells contributed to the anti-tumor activity induced by combination.

CD4 positive T, CD8 positive T or NK cell-depleted naive mice were inoculated intradermally with OV-HM cells (1×10^6 cells/mouse). (A) Tumor-bearing mice treated with PBS, (B) intact mice, (C) NK cell-depleted mice, (D) CD4 positive T cell-depleted mice, (E) CD8 positive T cell-depleted mice, (F) CD4 positive T cell and CD8 positive T cell-depleted mice treated with AdRGD-IL-12 and AdRGD-CCL27 at a ratio of 9:1 (in total 2×10^7 PFU/mouse).

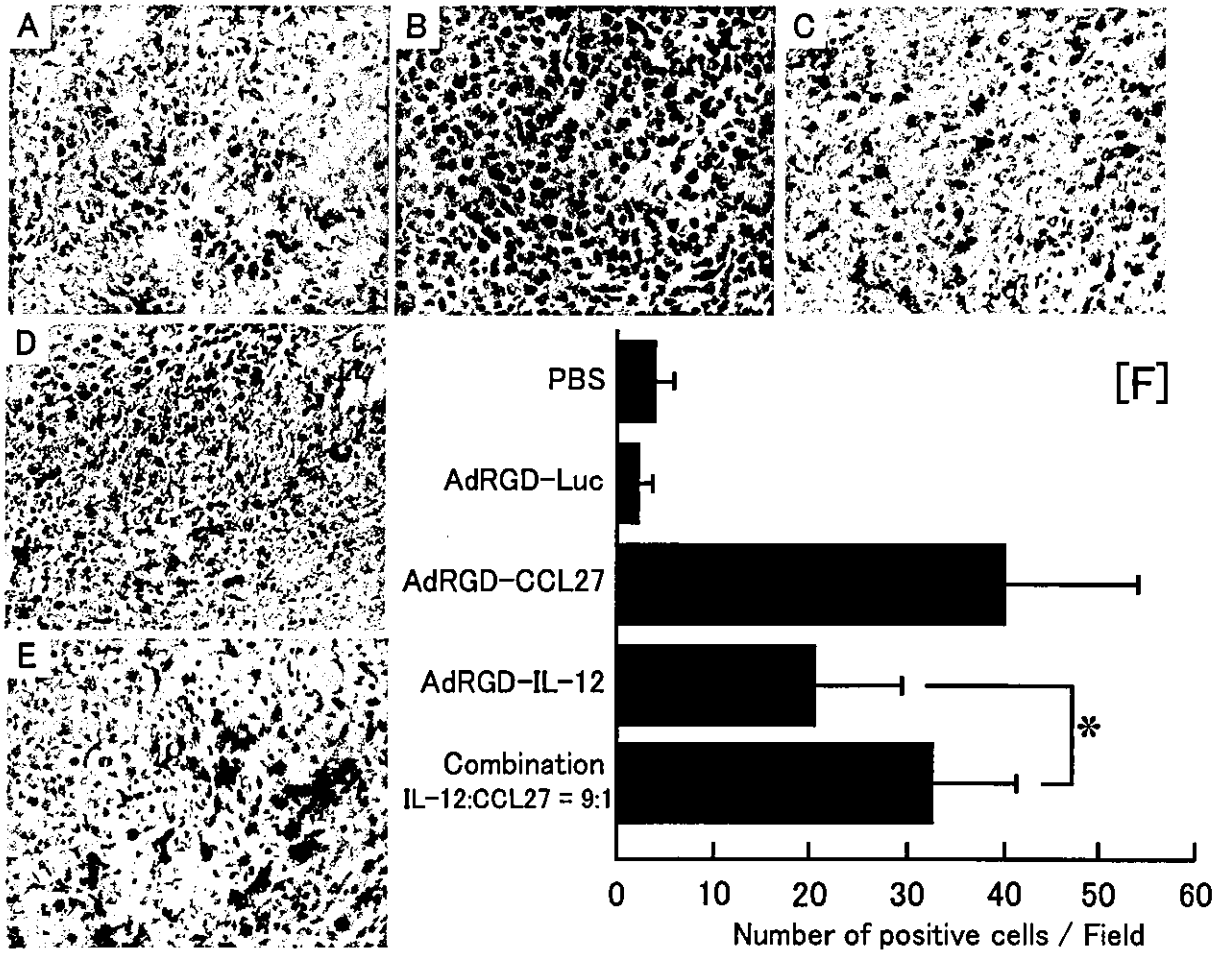

Fig.5 CD3 positive lymphocyte infiltrate into OV-HM tumor.
Immunohistochemical analysis was utilized to determine lymphocytes infiltrated into tumors. When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administration of (A) PBS, (B) AdRGD-Luc, (C) AdRGD-CCL27, (D) AdRGD-IL-12 or (E) combination (AdRGD-IL-12:AdRGD-CCL27=9:1). The tumor nodules were harvested, embedded in the O.C.T. compound, and stored at -80℃. Frozen thin sections of the nodules were fixed and stained for CD3 positive T cells using the method described above. (F) The number of immunostained cells were counted under light microscope with ×400 magnihication. For countinf positive cell number infiltrated into tumor tissue, six fields were randomly selected. Statistical analysis was carried out by Student's t-test. *; < 0.05

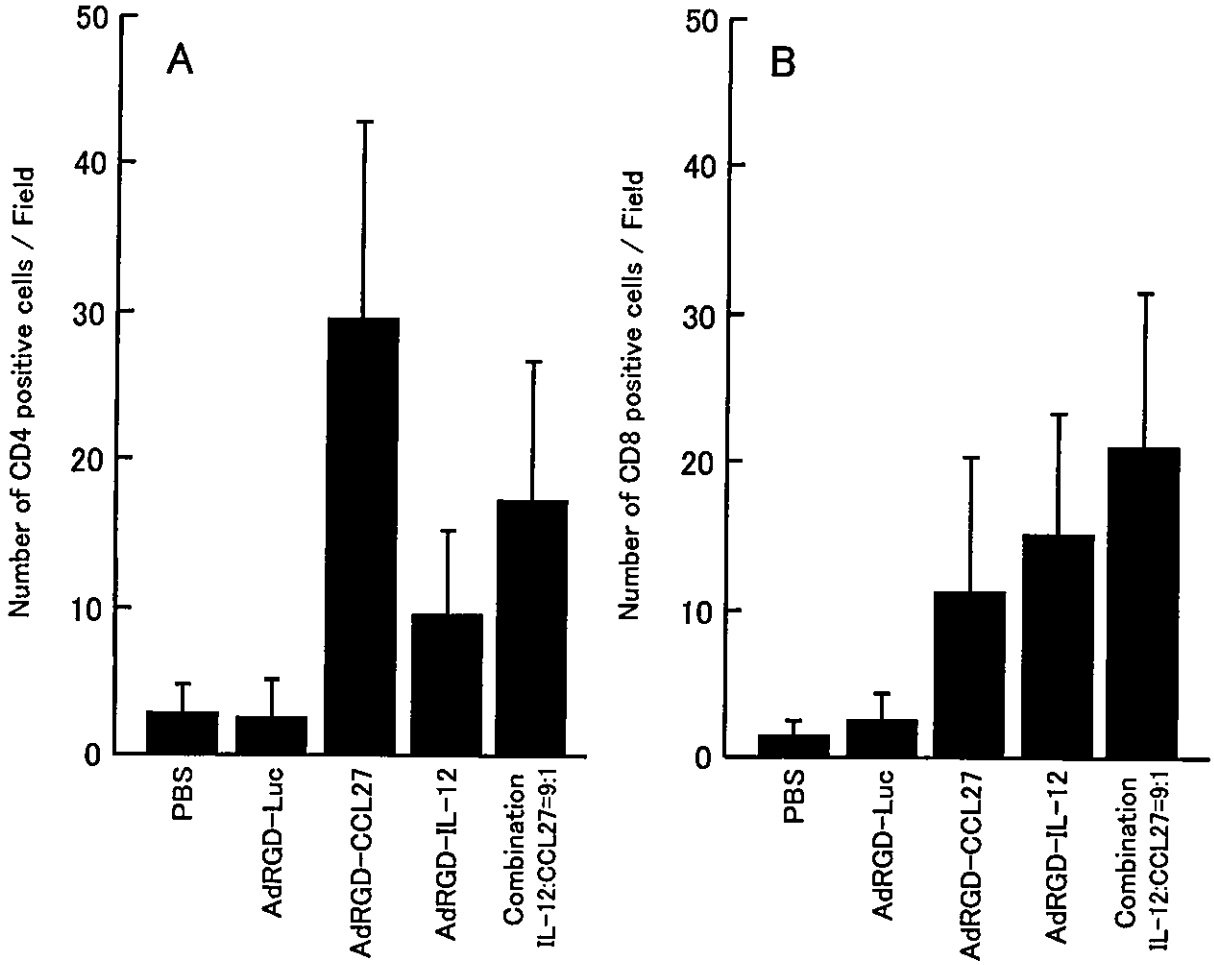

Fig. 6 CD4 or CD8 positive lymphocyte infiltrate into OV-HM tumor.
When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administration of AdRGD-CCL27, AdRGD-IL-12 or combination. The tumor nodules were harvested, embedded in the O.C.T. compound., stored at -80℃. Frozen thin sections of the nodules were fixed and stained for CD4 (A) or CD8 (B)-positive cells using the method described above. The number of immunostained cells were counted under light microscope with × 400 magnification. For counting the positive cell number infiltrated into tumor tissue, six fields were randomly selected.

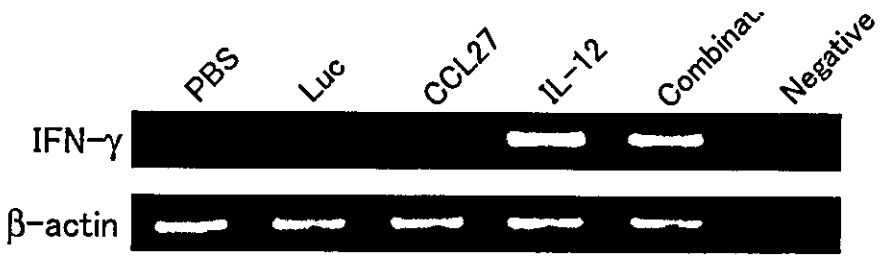

Fig. 7 RT-PCR analysis of murine IFN-γ in indicated adenovirus vectors injected OV-HM tumor nodules.
Total RNA was extracted from OV-HM tumor nodules, and then RT-PCR was performed to amplify the mRNA levels of mouse IFN-γ (379bp) and β-actin (514bp). PCR products were visualized by ethidium bromide staining after electrophoresis on an agarose gel. Negative was performed PCR using water as template.

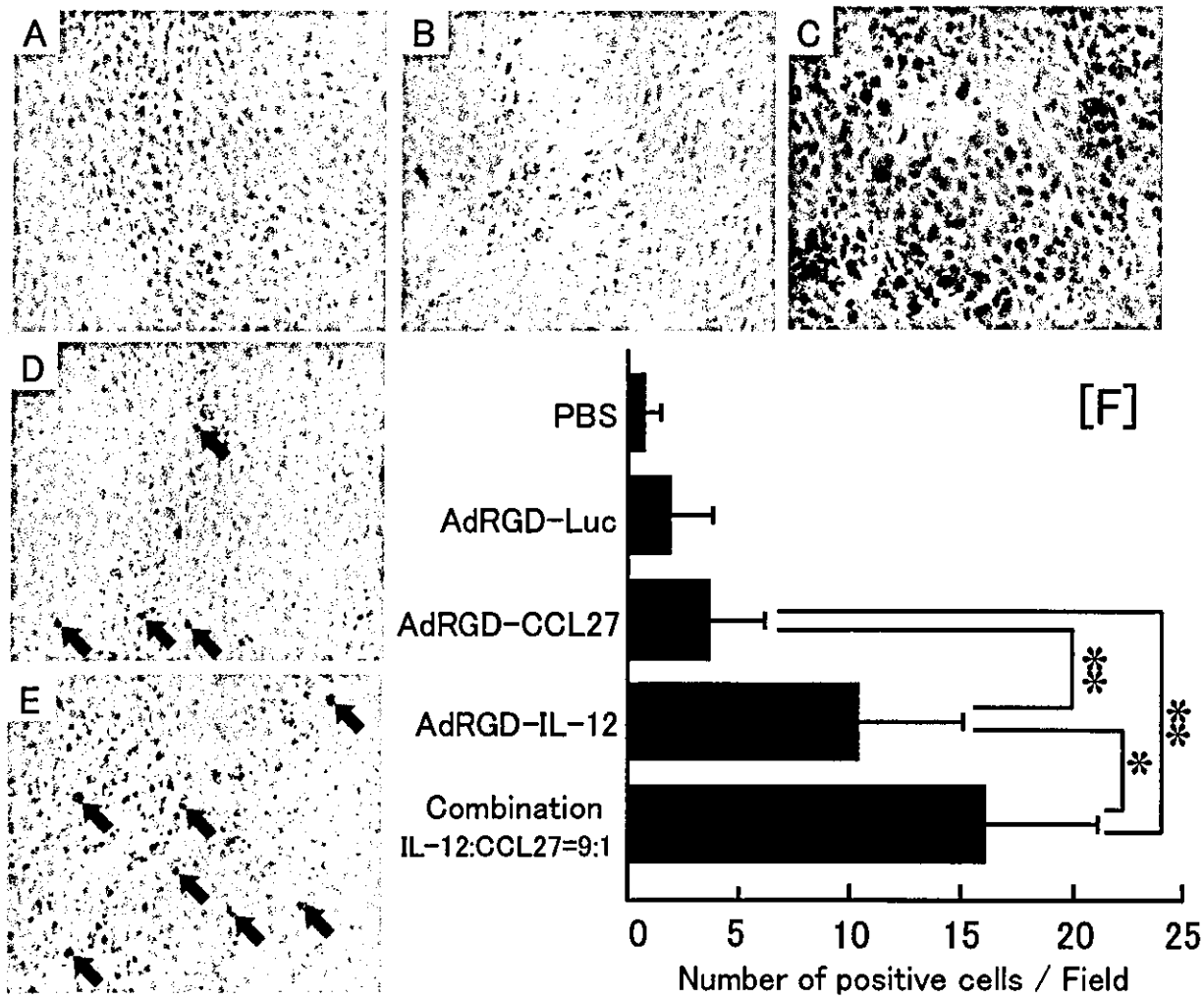

Fig. 8 Perforin positive cells infiltrate into OV-HM tumor.
When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administrations of (A) PBS, (B) AdRGD-Luc, (C) AdRGD-CCL27, (D) AdRGD-IL-12 and (E) combination (AdRGD-IL-12:AdRGD-CCL27=9:1). The tumor nodules were harvested, embedded in the O.C.T. compound, and stored at -80°C. Frozen thin sections of the nodules were fixed and stained for perforin-positive cells using the method described above. The number of immunostained cells were counted under light microscope with × 400 magnification. For counting the positive cell number infiltrated into tumor tissue, 6 fields were randomly selected. (F) Quantitation of perforin-positive cells in treated tumors. Statistical analysis was carried out by Student's t-test. *; < 0.05, **; < 0.01

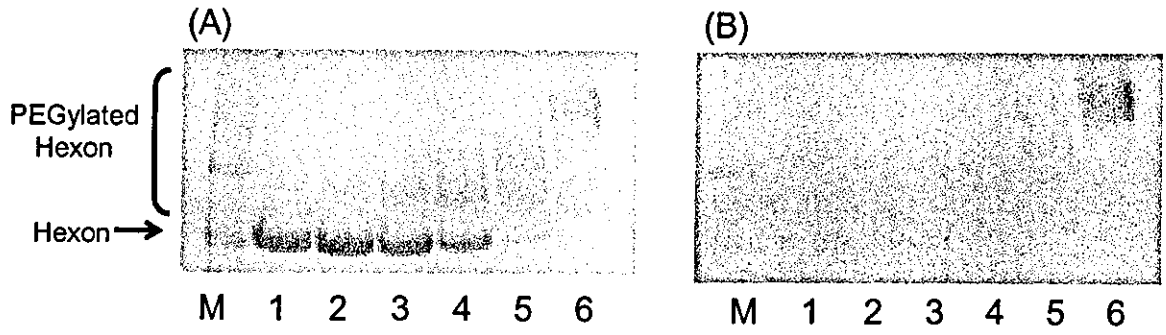

Fig. 9 SDS-PADE analysis of PEGylated adenovirus vectors.
Comparison of two SDS-gels (A, B) that were run under identical conditions and loaded as follows : lane M, protein markers ; lane 1, Ad : PEG=1:0 (unmodified-Ad) ; lane 2, 1:25 ; lane 3, 1:100 ; lane 4, 1:400 ; lane 5, 1:1600 ; lane 6, 1:6400. (A) The gel was stained with Coomassie blue. (B) The gel was stained for PEG using barium iodide.

Table 2 Relationship between degree of PEGylated-Hexon and adenovirus vector size

Ratio (Ad:PEG)*	PEG modification ratio (%)	Vector size (nm)	Serum half-life (min)
1:0	0	113.3±0.76	1.6
1:25	10	120.6±0.64	1.8
1:100	34	123.8±0.98	1.8
1:400	61	128.5±1.25	5.0
1:1600	89	137.6±0.91	12.0
1:6400	100	148.2±1.48	78.6

* ; Amount of PEG to lysine residue of adenovirus vector capsid protein (mol : mol)

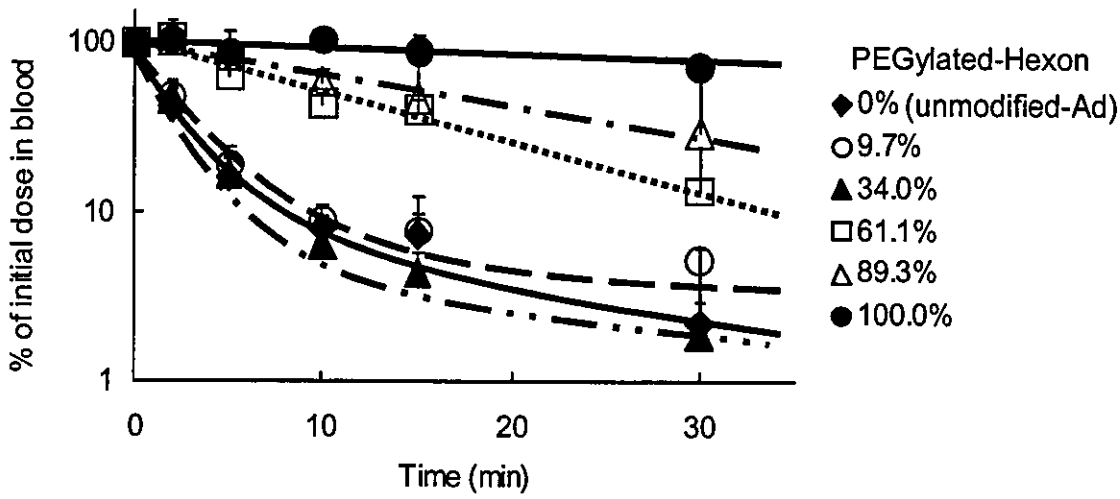

Fig. 10 Pharmacokinetics of PEGylated adenovirus vectors.
Normal female BALB/c mice were administrated intravenously with $1x10^{10}$ particles of unmodified-Ad or PEG-Ads. Blood samples were drawn at different times. The concentration of adenovirus vectors in serum was quantitated with southern blot method. A standard curve was made for each PEG-Ads. Each point was represented as mean ±S.D. (n=4).

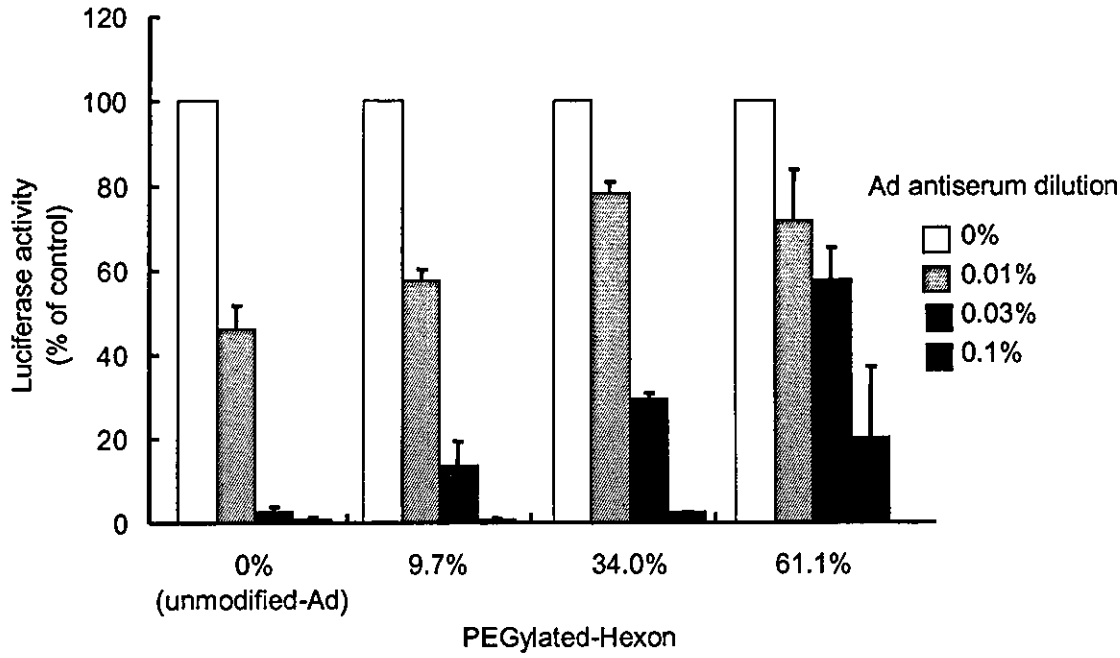

Fig. 11 Transduction of A549 cells by PEGylated adenovirus vectors in the presence or absence of adenovirus vectors antiserum.
A549 cells ($1x10^4$ cells) were tranceduced with 1000 particles/cell of unmodified-Ad or PEG-Ads in the presence or absence of Ad antiserum respectively. Luciferase expression was measured after 24 hr. Each point was represented as mean ±S.D. (n=3).

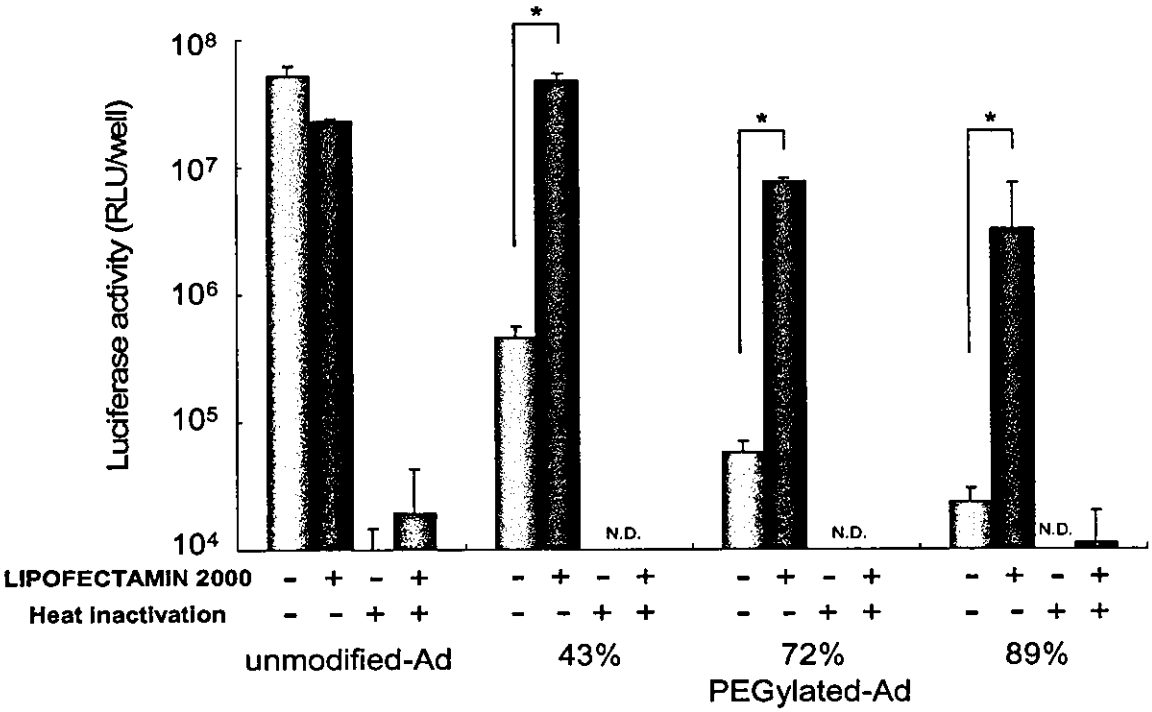

Fig. 12 Transduction efficiency of PEGylated adenovirus vectors into A549 cells in the presence or absence of LIPOFECTAMINE 2000.
A549 cells ($2x10^4$ cells) were transduced with 1000 particles/cell of unmodified or PEGylated Ad-Luc in the presence or absence of 20 μ g/ml of LIPOFECTAMINE 2000. After 4 hr, the virus solution was replaced with fresh medium, and the cells were incubated for 24 hr. Luciferase expression was measured. Each point represents the mean ± S.D. (n=3). * $P<0.05$ (*t-test*).

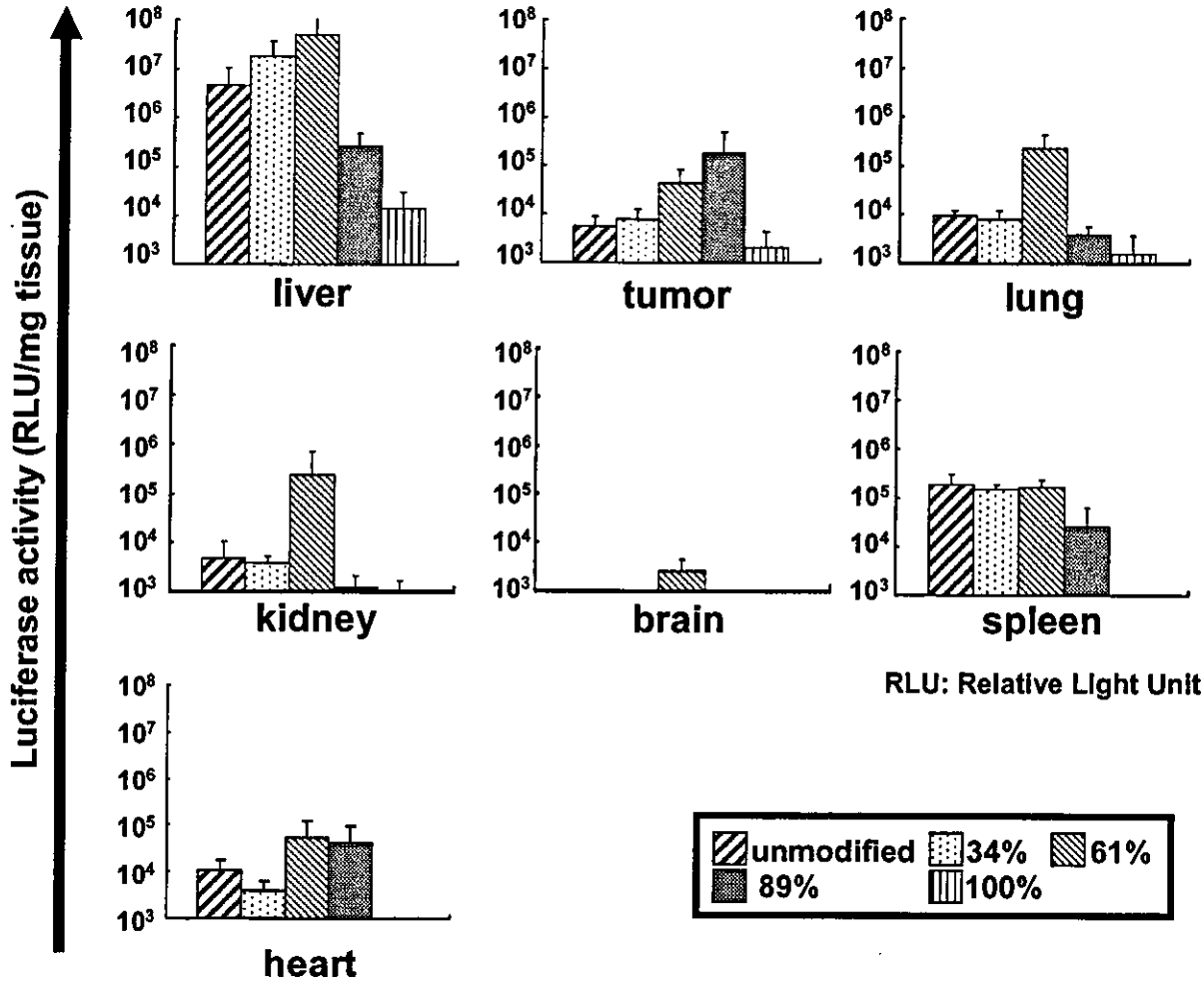

Fig. 13 ***In vivo*** **gene expression pattern of PEG-Ad after i.v. administration into mice.** 2×10^6 Meth-A fibrosarcoma tumor cells were inoculated intradermally and 10^{10} particles of unmodified or PEGylated Ad-Luc were injected intravenously after approximately one week. After 2 days, organs were harvested and homogenized with buffer. Luciferase activity was then measured using the kit according to the manufacture's instructions.(n=4).

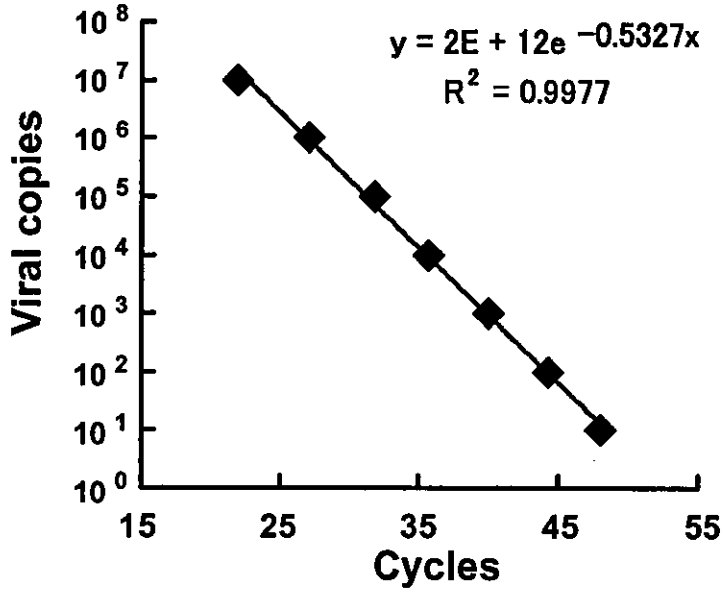

Fig. 14 Standard curve of TaqMan Real-time PCR

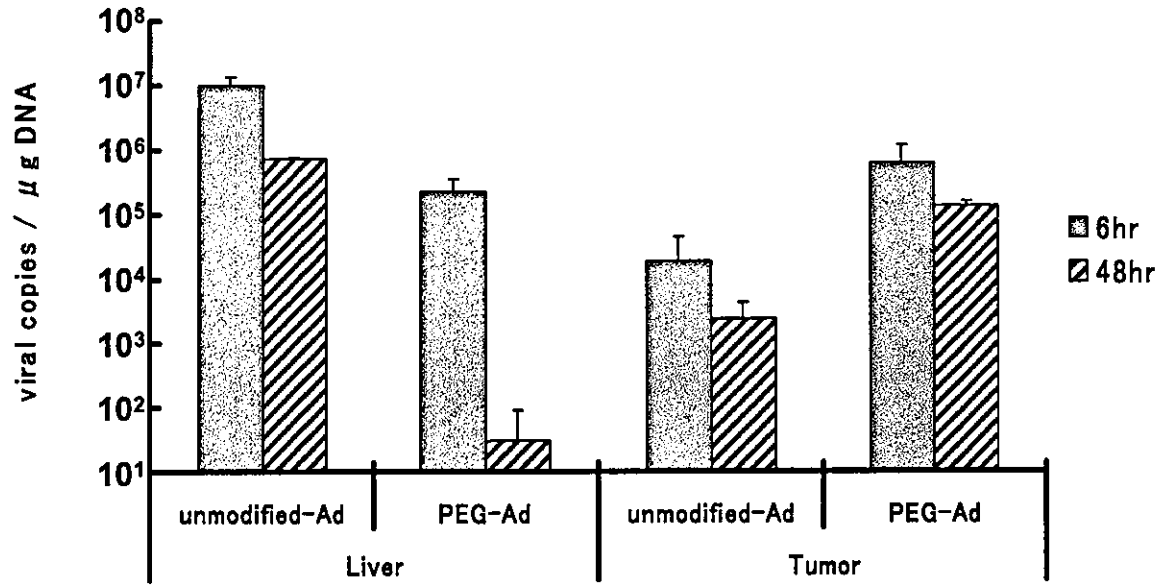

Fig. 15 Accumulation of Ad particles in tumor, and reduction in liver induced by PEGylation.
Real-time PCR was carried out for detecting viral particles existence in tumor and liver 6, 48 h after systemically administration of 1 x 10^{11} VP of both unmodified-Ad and PEGylated Ad (89% of modification ratio).

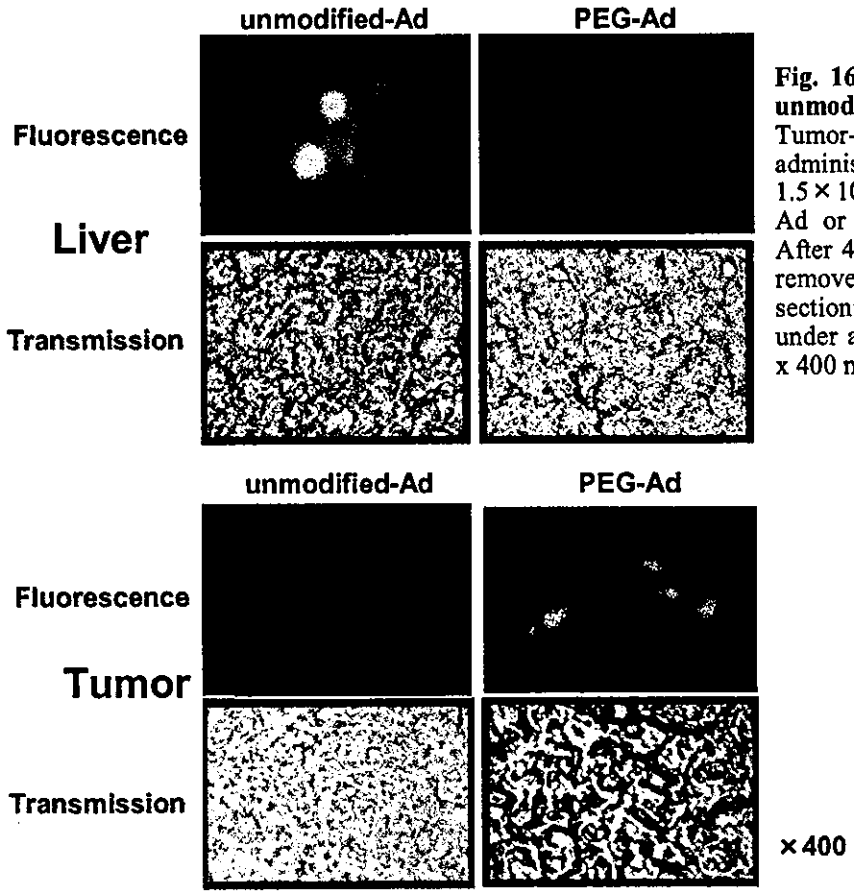

Fig. 16 EGFP gene expression of unmodified-Ad and PEG-Ad.
Tumor-bearing mice were administrated intravenously with 1.5×10^{11} particles of unmodified-Ad or PEG-Ad expressing EGFP. After 48 hr, livers and tumors were removed, processed for frozen sectioning. EGFP was observed under a fluorescence microscopy at x 400 magnification.

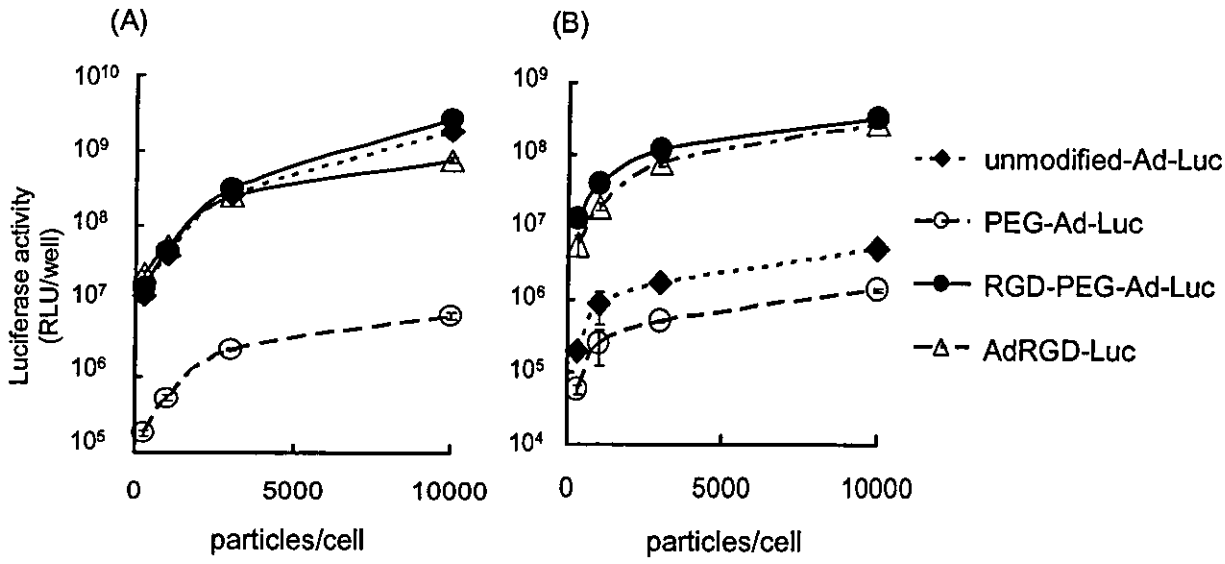

Fig. 17 Transduction of A549 cells and B16BL6 cells by RGD-PEGylated adenovirus vectors
(A) A549 cells and (B) B16BL6 cells ($2x10^4$ cells) were transfected with 300, 1000, 3000 or 10000 particles/cell of Ad, PEG-Ad-Luc, RGD-PEG-Ad-Luc or AdRGD-Luc respectively. Luciferase expression was measured after 24 hr. Each point was represented as mean ±S.D.

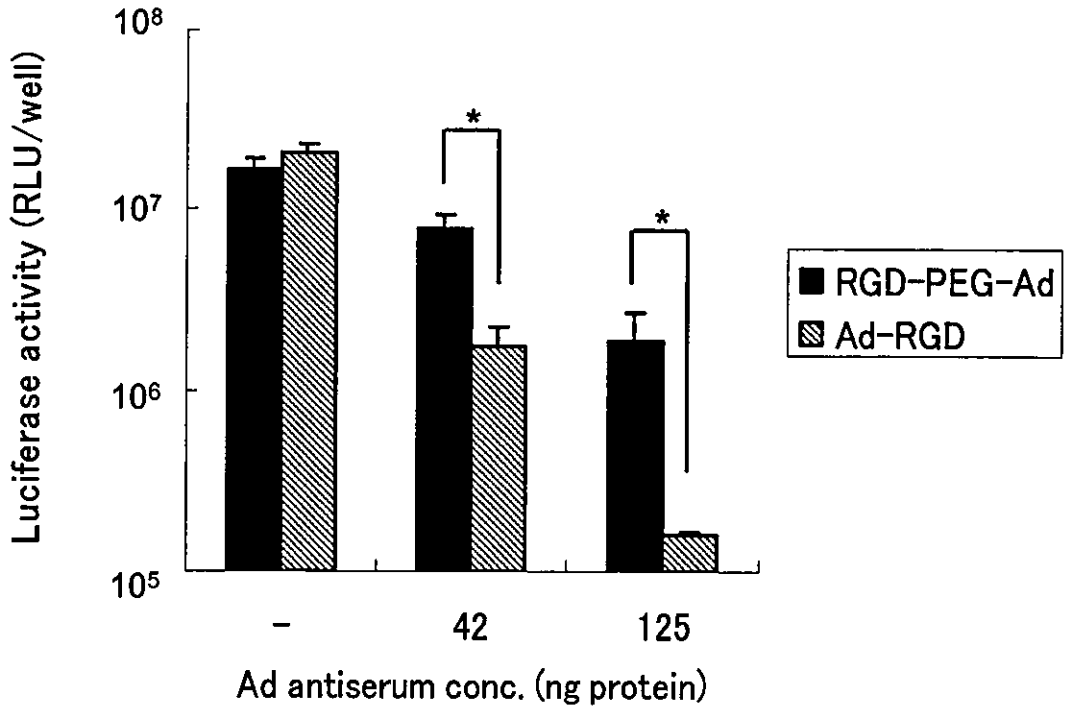

Fig. 18 Transduction of B16BL6 cells by RGD-PEGylated adenovirus vectors in the presence or absence of adenovirus vectors antiserum.
B16BL6 cells ($2x10^4$ cells) were tranceduced with 1000 particles/cell of RGD-PEG-Ad or AdRGD in the presence or absence of Ad antiserum respectively. Luciferase expression was measured after 24 hr. Each point was represented as mean ±S.D. (n=3).

研究成果の刊行に関する一覧表

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HUMAN GENE THERAPY 15:1034–1044 (November 2004)

Review

Targeted Adenovirus Vectors

HIROYUKI MIZUGUCHI[1] and TAKAO HAYAKAWA[2]

ABSTRACT

Recombinant adenovirus (Ad) vectors continue to be the preferred vectors for gene therapy and the study of gene function because they are relatively easy to construct, can be produced at high titer, and have high transduction efficiency. However, in some applications gene transfer with Ad vectors is less efficient because the target cells lack expression of the primary receptor, coxsackievirus and adenovirus receptor (CAR). Another problem is the wide biodistribution of vector in tissue following *in vivo* gene transfer because of the relatively broad tissue expression of CAR. To overcome these limitations, various approaches have been developed to modify Ad tropism. In one approach, the capsid proteins of Ad are modified, such as with the addition of foreign ligands or the substitution of the fiber with other types of Ad fiber, in combination with the ablation of native tropism. In other approaches, Ad vectors are conjugated with adaptor molecules, such as antibody and fusion protein containing an anti-Ad single-chain antibody (scFv) or the extracellular domain of CAR with the targeting ligands, or chemically modified with polymers containing the targeting ligands. In this paper, we review advances in the development of targeted Ad vectors.

INTRODUCTION

ADENOVIRUS VECTORS have been expected to play a prominent role in gene therapy because of their extremely high transduction efficiency. However, one of the hurdles confronting gene transfer by adenovirus (Ad) vectors is their inefficient transduction to target cells lacking sufficient expression of the coxsackievirus and adenovirus receptor (CAR), the primary receptor; such cells include many advanced tumor cells, skeletal muscle cells, smooth muscle cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, and so on. A high dose of vector is required to achieve efficient gene transfer to these cell types. This in turn increases unwanted side effects, such as vector-associated immunogenic toxicities.

Another hurdle confronting Ad vector-mediated gene transfer is their nonspecific distribution in tissue after *in vivo* gene transfer because of the relatively broad expression of CAR, α_v integrin (the secondary receptor), and heparan sulfate (the third receptor). This property imposes an increased risk of toxicity due to vector dissemination to nontargeted cells, such as antigen-presenting cells (e.g., macrophages and dendritic cells). This occurs even when Ad vectors are locally administered to the tissue of interest. Vector targeting to a specific tissue or cell type would enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

Several approaches have been developed to overcome these hurdles, including genetic modification of Ad capsid proteins, such as fiber, penton base, hexon, and protein IX (pIX), and conjugation-based modification of virus such as antibody or bispecific fusion protein, and chemical modification by polymers containing the targeting ligands (Fig. 1). To improve gene transfer efficiency, modification of tropism is required. To target gene transfer, both the ablation of natural tropism and introduction of cell-specific tropism are required. In this paper we review approaches to developing targeted Ad vectors.

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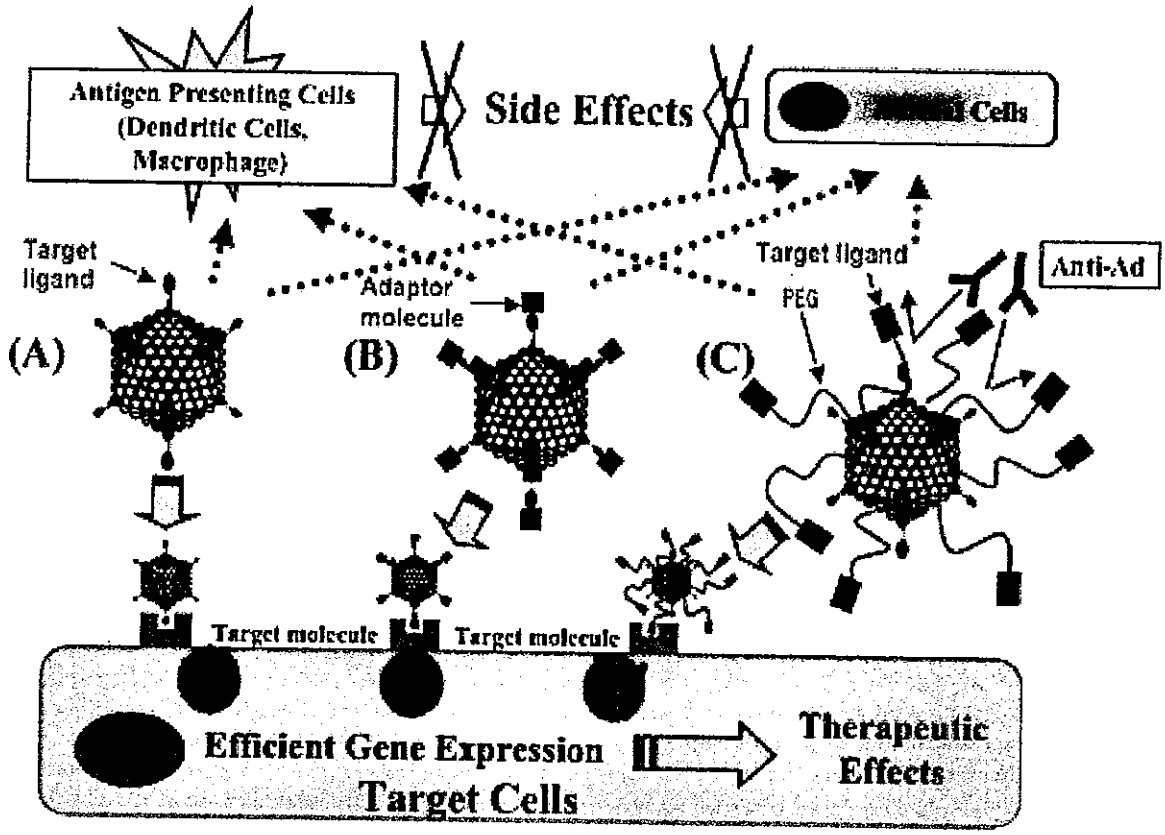

FIG. 1. Three approaches to developing targeted Ad vectors. (A) Genetic modification of virus capsid. (B) Modification by the use of adaptor molecules. (C) Chemical modification by polymers with ligands.

KINETICS OF ADENOVIRUS VECTOR-MEDIATED GENE TRANSFER *IN VIVO*

Important determinants of virus clearance from the bloodstream include interactions between viral components and cellular receptors, virion size, net charge of the viral particle, and anatomical barriers, such as tightness of the basal membrane of endothelial cells. Understanding factors that impact on the kinetics of blood clearance and the biodistribution of Ad vectors would be beneficial to advancing their application as therapeutic agents.

Systemically administered Ad vectors are rapidly cleared from the blood of mice, with a half-life of less than 3 min (Alemany *et al.*, 2000; Koizumi *et al.*, 2003a; Sakurai *et al.*, 2003b). Liver Kupffer cells play a central role in clearing Ad genomes from the bloodstream (Lieber *et al.*, 1997; Wolff *et al.*, 1997; Worgall *et al.*, 1997). Activated Kupffer cells (and monocytes and resident macrophages) release proinflammatory cytokines/chemokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interferon γ-inducible protein 10 (IP-10), and RANTES (regulated on activation, normal T cell expressed and secreted), causing the activation of an innate immune response (Liu and Muruve, 2003). It has been proposed that a low dose of Ad vectors ($\sim 10^{10}$ vector particles) is rapidly sequestered by Kupffer cells (nonparenchymal cells), whereas higher doses of Ad vectors are delivered into both Kupffer cells and parenchymal cells, leading to a nonlinear dose response in hepatic transgene expression (Tao *et al.*, 2001). At a dose of 3.0×10^{10} vector particles, Ad vectors are likely to be equally distributed to Kupffer and parenchymal cells (Koizumi *et al.*, 2003a).

The liver directivity of the systematically administered Ad vectors can also be applied when local administration of the vectors is performed. Even if the Ad vector is injected into local tissues such as tumors, large amounts of vector are distributed into the bloodstream and targeted into the liver, causing unwanted side effects (Mizuguchi and Hayakawa, 2002b; Okada *et al.*, 2003). The process of Ad vector-mediated liver transduction is influenced by interactions between viral components and cellular receptors (discussed in Truly Targeted Adenovirus Vectors, below), the size of the sinusoidal fenestrae (Fechner *et al.*, 1999; Lievens *et al.*, 2004), and the complement system (Zinn *et al.*, 2004). Lievens *et al.* showed that Ad vector-mediated liver transduction in Dutch Belt rabbits, with 124-nm sinusoidal fenestrae, is significantly higher than that in New Zealand White rabbits, which have 108-nm sinusoidal fenestrae, and Fauve de Bourgogne rabbits with 105-nm sinusoidal fenestrae (Lievens *et al.*, 2004). The increase in sinusoidal fenestrae to 123 nm in New Zealand White rabbits by the intraportal injection of sodium decanoate enhances Ad vector-mediated liver transduction, confirming that the size of the sinusoidal

fenestrae is an important determinant for liver transduction (Lievens *et al.*, 2004). For targeting Ad vector to extrahepatic tissues, it is important to avoid distribution into parenchymal and nonparenchymal (Kupffer) cells of the liver as well as other tissues, such as spleen.

APPROACHES TO DEVELOPING TARGETED ADENOVIRUS VECTORS

Genetic modification of the virus capsid

Modification of virus tropism. Modification of the fiber proteins has been used to successfully overcome barriers to transduction due to a paucity of CAR. Two approaches have been used for this purpose. One is the addition of foreign peptides to the HI loop or C terminus of the fiber knob (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998; Mizuguchi *et al.*, 2001; Koizumi *et al.*, 2003b). Another is the substitution of fibers derived from other Ad serotypes, which bind to receptor molecules other than CAR (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Mizuguchi and Hayakawa, 2002a). Both approaches allow Ad tropism to be expanded (or changed) via binding of the modified fiber protein with a different cellular receptor.

Expanded and higher rates of gene transfer have been reported on the basis of the use of mutant fiber proteins containing an Arg-Gly-Asp (RGD) peptide (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Hidaka *et al.*, 1999; Mizuguchi *et al.*, 2001) or a stretch of lysine residues (KKKKKKK [K_7] peptide) (Wickham *et al.*, 1997; Hidaka *et al.*, 1999), which target α_v integrins or heparan sulfates to the cellular surface, respectively. The RGD peptide has been displayed in the HI loop or C terminus of the fiber knob, whereas the K_7 peptide has been displayed at the C terminus of the fiber knob. There have also been reports of inserting the peptides into the HI loop of the fiber knob, including those discovered by phage display library to show high affinity for vascular endothelial cells (Nicklin *et al.*, 2000), cancer cells (Nicklin *et al.*, 2003), transferrin receptor (Xia *et al.*, 2000), and vascular smooth muscle cells (Work *et al.*, 2004).

Altered vector tropism was reported by substitution of the Ad type 5 (Ad5) fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad35, and others (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Rea *et al.*, 2001; Stecher *et al.*, 2001; Mizuguchi and Hayakawa, 2002a). Most Ad serotypes belonging to the subgroups A, C, D, E, and F use CAR as the initial receptor for the virion (Roelvink *et al.*, 1998), whereas Ad serotype B uses other molecules for infection (Roelvink *et al.*, 1998; Amberg *et al.*, 2000a,b; Law and Davidson, 2002; Burmeister *et al.*, 2004). Ad8, Ad19, and Ad37, which belong to serotype D, use sialic acids as the primary receptor (Arnberg *et al.*, 2000a,b; Burmeister *et al.*, 2004). CD46, CD80, and CD86 were identified as cellular receptor(s) of Ad belonging to subgroup B, including Ad3, Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50 (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Short *et al.*, 2004). Human CD34-positive cells, dendritic cells, synoviocytes, vascular endothelial cells (ECs), and smooth muscle cells (SMCs), which were poorly transfectable by conventional Ad vectors, were efficiently transfected by fiber-substituted Ad vectors (Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Okada *et al.*, 2001; Rea *et al.*, 2001). Mercier *et al.* described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing the junctional adhesion molecule 1 (JAM1) (Mercier *et al.*, 2004).

When modified Ad vectors are injected locally into target tissue expressing corresponding receptors, the affinity of the vector for the cells increases, thereby resulting not only in higher transduction efficiency, but also in decreased vector dissemination. We reported that the intratumoral administration of luciferase-expressing Ad vectors containing the RGD peptide in the HI loop of the fiber knob resulted in nearly 40 times more transgene production in tumor, but 8 times less transgene expression in liver in the B16 mouse melanoma model as compared with conventional Ad vectors (Mizuguchi and Hayakawa, 2002b).

Other candidate locations for insertion of foreign ligands into the Ad capsid are the pIX, the penton base, and the hypervariable region (HVR) 5 of hexon loop L1 (Wickham *et al.*, 1995; Vigne *et al.*, 1999; Dmitriev *et al.*, 2002; Vellinga *et al.*, 2004). Among them, pIX seems to be the most promising. pIX is a minor structural protein that is contained in the Ad virion, and enhances the structural integrity of the particles by stabilizing hexon–hexon interaction (Ghosh-Choudhury *et al.*, 1987; Furcinitti *et al.*, 1989). It also plays a role in transcriptional activity and nuclear reorganization (Rosa-Calatrava *et al.*, 2001). Foreign ligands are displayed at the C terminus of the pIX of Ad (Dmitriev *et al.*, 2002). The attractive characteristics of ligand insertion into the pIX region is that the C terminus of pIX tolerates the insertion of large peptides. By incorporation of the pIX–green fluorescent protein (GFP) fusion protein, a fluorescent Ad was generated (Le *et al.*, 2004; Meulenbroek *et al.*, 2004). The insertion of higher affinity ligands such as single-chain antibodies (scFv) would be ideal, although generating such Ad vectors might be difficult because of impaired assembly of complex scFv–pIX fusion proteins in the nucleus. One problem with pIX fusions is that Ad pIX resides below the top of the hexon capsomer, within the core of the virus. This problem was circumvented by incorporating an α-helical spacer into the ligand–pIX fusion protein so as to lift the ligand and expose it to the surface of the capsid (Vellinga *et al.*, 2004). However, Ad vectors containing the RGD motif in the C terminus of pIX with α-helical spacers are likely to be less efficient than Ad vectors containing the RGD motif in the HI loop of the fiber knob (Vellinga *et al.*, 2004). Additional modification may be required for improved efficacy and specificity of retargeting.

Several groups have developed an Ad vector from an entire Ad35, and have demonstrated higher transduction efficiency for the Ad35 vector into human CD34-positive cells and dendritic cells compared with the conventional Ad5 vector (Gao *et al.*, 2003; Sakurai *et al.*, 2003a,b; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003). In addition, Ad35 vectors have the advantage of evading humoral immune responses against Ad5. However, fiber-substituted Ad5 vectors containing fiber proteins of another serotype do not circumvent the immune response against Ad5 (Gall *et al.*, 1996; Ophorst *et al.*, 2004), because hexon is the major target of host-neutralizing antibodies in Ad5 infec-

tion (Gall *et al.*, 1996, 1998; Roy *et al.*, 1998). The Ad35 vector would be an effective alternative for use in persons with neutralizing antibodies in Ad5, and in the second injection when the Ad5 vector is used in the first injection of *in vivo* gene therapy.

Truly targeted adenovirus vectors. Although modifications described above yield Ad vectors with greatly improved transduction to many cells lacking in CAR expression, when systemically administered, vector dissemination, resulting in accumulation in liver, is unavoidable. To create a strictly targeted Ad vector, two basic requirements must be met. The first is construction of vectors that abolish natural viral tropism. The second is identification and incorporation of a foreign ligand with high affinity for a specific cellular receptor into the capsid of Ad vectors.

The capsid proteins determine the tropism of Ad. Because the fiber knob binds with CAR, this interaction first must be abolished. Mutation of the AB, DE, or FG loop of the fiber knob has been reported to abolish the fiber–CAR interaction (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Roelvink *et al.*, 1999). These mutations of the fiber knob greatly reduce the transduction efficiency of Ad vectors to CAR-positive cells *in vitro*. In another strategy, Nakamura *et al.* replaced the tail, shaft, and knob domains of the Ad5 fiber with those of the Ad40 short fiber, which is hypothesized not to bind to any receptors (Nakamura *et al.*, 2003). In addition, interaction of the RGD motif of the penton base with α_v integrin must be abolished, although this interaction might be minor, at least *in vitro* (Mizuguchi *et al.*, 2002). The ablation of α_v integrin binding was accomplished by deletion of the RGD motif of the penton bases. Several articles reported that a single mutation of either the fiber knob or penton base does not change the biodistribution of Ad vectors in mice after *in vivo* injection (Alemany and Curiel, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002), whereas double mutation reduces liver transduction (Einfeld *et al.*, 2001; Koizumi *et al.*, 2003a), although two groups showed that double mutation also does not reduce liver transduction (Martin *et al.*, 2003; Smith *et al.*, 2003b). The reason for this discrepancy is unclear. However, Nicol *et al.* reported that combining fiber knob and penton base mutations reduces liver transduction by 509-fold in rats, an effect not observed in parallel experiments in mice (Nicol *et al.*, 2004). Subtle differences among the vectors, such as differences in mutated amino acids, experimental animal strains used, or injected doses, might have caused these discrepancies. Furthermore, the fiber shaft domain of Ad5 was reported to be involved in accumulation in the mouse liver of systemically administrated Ad vectors (Nakamura *et al.*, 2003; Smith *et al.*, 2003b), possibly because of the interaction of the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of Ad5 with heparan sulfate (Smith *et al.*, 2003b). This effect was also observed in nonhuman primate (cynomolgus monkey) models (Smith *et al.*, 2003a). According to our data, triple mutation of

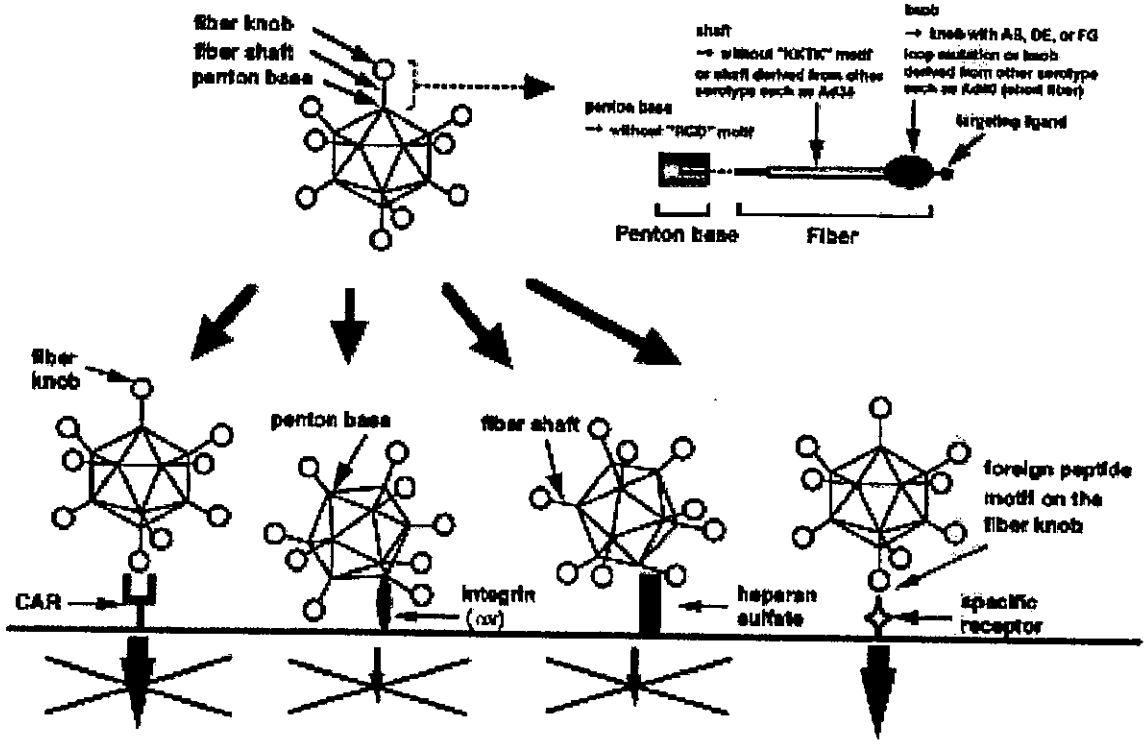

FIG. 2. Schematic diagram of Ad vectors targeted by the genetic approach. The CAR-, α_v integrin-, and heparan sulfate-binding activities of the Ad capsid are completely ablated by mutations in the fiber knob, the fiber shaft, and the penton base, respectively (Koizumi *et al.*, 2003a; Nicol *et al.*, 2004). Targeting ligands should be incorporated in the virus capsid, such as the fiber, the penton base, the hexon, or pIX. The resulting targeted Ad vectors transduce cells via the incorporated foreign ligand-dependent, CAR-, α_v integrin-, and heparan sulfate-independent pathway.

the fiber knob, shaft, and penton base mediated levels of liver transduction more than 30,000-fold lower than that of conventional Ad vectors (Koizumi *et al.*, 2003a). This vector contains a CAR-binding ablated mutant fiber knob derived from Ad5, a fiber shaft derived from Ad35 (the fiber shaft of Ad35 does not have the KKTK motif and is shorter than that of Ad5 [Ad5 fiber shaft, 6 β repeats; Ad5 fiber shaft, 22 β repeats]), a fiber tail derived from Ad5, and a mutant penton base of Ad5 without the RGD motif. Ad vectors, with mutations in two domains of the fiber knob, the fiber shaft, and the penton base, showed a level of liver transduction intermediate between that of conventional Ad vectors and the triple-mutant Ad vectors (Koizumi *et al.*, 2003a). Nicol *et al.* reached a similar conclusion in a rat model (Nicol *et al.*, 2004). Thus, Ad tropism would be determined by at least three factors: the fiber knob, the fiber shaft, and the RGD motif of the penton base (Figs. 1A and 2). Triple mutations, including the fiber knob, the fiber shaft, and the RGD motif of the penton base, should be preferable for the platform of targeted Ad vectors.

A detailed study on vector distribution to the liver, however, suggested that triple-mutant Ad vectors distribute to nonparenchymal cells to a similar extent as conventional vectors, and that both vectors are cleared rapidly from the bloodstream, having a half-life of less than 2 min (Koizumi *et al.*, 2003a). This nonparenchymal cell-mediated clearance might present an obstacle to the development of targeted Ad vectors that incorporate a foreign ligand into the viral capsid. One promising strategy to overcome this problem might be intraperitoneal, not intravenous, injection of the vector. Akiyama *et al.* reported that the intraperitoneal administration of CAR and integrin binding-ablated Ad vectors increases their persistence in the bloodstream, although the mechanism by which this occurs is unknown (Akiyama *et al.*, 2004). Extended release of the vector from the cavity might change its pharmacokinetics. More detailed study is needed to clarify nonparenchymal cell-mediated vector clearance. Lower clearance from the bloodstream may lead to increased delivery of the vector to the tissue of interest, if an appropriate targeting ligand is incorporated into the vector.

The identification of targeting ligands that are displayed in the capsid, such as fiber and pIX, is another challenge. A display library using filamentous phage is widely used for the identification of functional peptides for targeting. Although some success in identifying peptide ligands for the targeted Ad vectors was reported (Nicklin *et al.*, 2000; Xia *et al.*, 2000; Work *et al.*, 2004), most peptides that are identified by phage display libraries are not functional when they are displayed in the fiber knob of Ad vectors. Foreign peptides inserted into the HI loop of the fiber knob are constrained at both the N and C termini, whereas peptides inserted at the C terminus of the fiber knob are constrained only at the N terminus. In contrast, peptides identified by filamentous phage display library are constrained only at the C terminus, when the peptides are displayed as a fusion protein with the product of gene III of the phage. The lack of efficacy of peptides inserted in the fiber knob could be due to this difference when the peptides are identified. Furthermore, the lack of efficacy would be dependent on conformational changes after ligation of the peptide to the fiber knob. To overcome these limitations, Pereboev *et al.* employed a modified filamentous phage-displayed system, pJuFo, which was originally designed to display C-terminal protein fragments (Pereboev *et al.*, 2001). They developed a system for displaying peptides in the context of the fiber knob on the surface of the phage. A display system based on phage λ, which expresses a functional Ad fiber knob on the surface, was also developed (Fontana *et al.*, 2003). By using these systems, Ad vectors containing novel peptide ligands were generated, transducing NIH3T3 and dendritic cells at 100- to 1000-fold higher efficiency than conventional vectors (Fontana *et al.*, 2003). The development and evaluation of the next generation of targeted vectors by incorporating the novel peptides into native tropism-ablated Ad vectors is expected. In the case of the adeno-associated virus (AAV) vector, a method for incorporating random small peptides in the viral capsid has been developed (Muller *et al.*, 2003). This type of screening for ligands might be useful for targeted Ad vector, although the creation of an Ad library with wide diversity is a challenge.

Propagation of modified Ad vectors that no longer bind with cellular receptors (CAR, α_v integrin, and heparan sulfate) requires a special packaging cell line. Two types of packaging cell lines have been reported. One utilizes 293 cells modified to express an artificial receptor molecule (Douglas *et al.*, 1999; Roelvink *et al.*, 1999) that should not have any natural analogs, such as the anti-His single-chain antibody (scFv) and anti-hemagglutinin (HA) scFv. The other approach is to use 293 cells expressing Ad5 fiber protein (Fiber-293 cells) (Von Seggern *et al.*, 1998; Legrand *et al.*, 1999; Koizumi *et al.*, 2003a). In the case of cell lines expressing anti-His scFv, a His tag sequence has been introduced into the C-terminal region of the fiber knob in Ad vectors (Douglas *et al.*, 1999), whereas in the case of cell lines expressing anti-HA scFv, an HA tag sequence has been introduced into the HI loop of the fiber knob or the penton base instead of the RGD motif (Roelvink *et al.*, 1999). Modified Ad vectors are generated by interaction of the tag sequence in the virus with the scFv against the tag sequence on the cells. When the modified Ad vectors are propagated in Fiber-293 cells, wild-type fibers are incorporated in the virus during amplification, resulting in the virus containing both wild-type fibers and mutated fibers. This virus infects 293 (Fiber-293) cells via the wild-type fiber. At the final stage of viral amplification, mutated Ad vectors are allowed to infect normal 293 cells. The recovered viruses should contain only mutant fiber proteins. When Fiber-293 cells have been used as packaging cell lines, either the HI loop or the C-terminal region of the fiber knob as well as the penton base can be used to display a foreign ligand on the vectors. This makes these cells advantageous over cell lines expressing anti-His scFv or anti-HA scFv. In both methods, modified vectors were generated to particle titers similar to that of conventional Ad vectors (Douglas *et al.*, 1999; Roelvink *et al.*, 1999; Koizumi *et al.*, 2003a).

Another strategy to ablate CAR binding by Ad vectors is to proteolytically remove the knob domain of Ad fibers via the insertion of a single factor Xa cleavage site in the fiber shaft, between the cellular ligand and knob domain (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004). As cellular ligands, the RGD peptide and a 58-residue oligopeptide termed the affibody, which binds specifically to the human IgG1 Fc domain, were introduced and ligand-mediated gene transfer was reported (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004).

Ad vectors in which the fiber protein was replaced with phage T4 fibritin were also developed (Krasnykh *et al.*, 2001;

Belousova *et al.*, 2003; Papanikolopoulou *et al.*, 2004). In these vectors, structural similarity between the Ad fiber and bacteriophage T4 fibritin proteins was used, and the fiber shaft and knob domains were replaced with T4 fibritin and a receptor-binding ligand. The human CD40 ligand was functionally displayed in the chimeric fiber of the Ad vectors (Belousova *et al.*, 2003). This approach seems to overcome structural conflicts between the fiber and the targeting ligand.

As described above, several types of vector systems have been developed, using a genetic strategy. These vectors would provide a platform for future targeted Ad vector development. Future efforts should be directed toward novel ligands for specific tissue targeting.

Modification by the use of adaptor molecules

Retargeting of Ad infection can also be achieved through the use of bispecific or bifunctional adaptor molecules composed of an anti-fiber antibody fragment and a cell-binding component. Douglas *et al.* 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 shown to redirect, at high efficiency, the Ad infection of target cells via the folate receptor (Douglas *et al.*, 1999). The Fab fragment of the anti-fiber mAb has been utilized to conjugate with several other ligands including fibroblast growth factor 2 (FGF-2) (Goldman *et al.*, 1997; Sosnowski *et al.*, 1999), epidermal growth factor receptor (EGFR) (Miller *et al.*, 1998), and an anti-CD40 mAb fragment (Tillman *et al.*, 1999). Reynolds *et al.* succeeded in targeting pulmonary endothelial cells *in vivo* by the intravenous injection of Ad vectors complexed with bispecific antibody against the Ad fiber knob and angiotensin-converting enzyme (Reynolds *et al.*, 2000). In a similar strategy, the anti-Ad fiber knob scFv (Watkins *et al.*, 1997; Haisma *et al.*, 2000; Nettelbeck *et al.*, 2001) or the extracellular domain of CAR (Dmitriev *et al.*, 2000; Itoh *et al.*, 2003) was used as the attachment molecule with the virus. Fusion proteins or complexes of ligands with the anti-Ad fiber knob scFv or CAR were used as adaptor molecules (Fig. 1B).

Combination of the adaptor molecule and genetically modified capsids of the Ad vector has also been reported. The Fc-binding domain of staphylococcal protein A was genetically incorporated into the Ad fiber protein (Henning *et al.*, 2002; Korokhov *et al.*, 2003; Volpers *et al.*, 2003). Two studies incorporated the Fc-binding domain into either the HI loop or C terminus of the fiber knob (Korokhov *et al.*, 2003; Volpers *et al.*, 2003), whereas one study incorporated the Fc-binding domain into a knob-deleted fiber containing seven shaft repeats and an external trimerization motif (Henning *et al.*, 2002). Targeting components such as the antibody and fusion protein of the ligand with the Fc domain of immunoglobulin effectively bind to the modified Ad vectors, resulting in specific gene delivery. Because the target-specific ligands such as antibodies are simply changed in this system, these types of Ad vectors should be useful for systematic screening and detection of the target-specific ligands, as well as for therapeutic applications.

Metabolically biotinylated Ad vectors have been developed as another type of vector with adaptor molecule and genetically modified capsid. Barry and colleagues designed a system based on the fusion of a truncated form of the *Propionibacterium shermanii* 1.3S transcarboxylase domain (PSTCD), which functions as a biotin acceptor peptide (BAP) and is efficiently biotinylated by human holocarboxylase synthetase, to the C terminus of the Ad fiber protein (Parrott *et al.*, 2003) or the C terminus of the Ad pIX protein (Campos *et al.*, 2004). In this system, Ad vectors containing BAP are metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells, resulting in covalently biotinylated virions. Biotinylated Ad vectors are useful as a platform for avidin-based ligand screening and vector targeting by conjugating biotinylated ligands to the virus, using high-affinity tetrameric avidin. Their group performed ligand screening for dendritic cells, using biotinylated Ad vectors (Parrott *et al.*, 2003).

Theoretically, in all the approaches discussed above, any conjugates with one component directed against the Ad capsid (or modified capsid) and the second component directed against the cell surface protein can be applied to increase transduction of target cells. The advantage is that the natural tropism of the fiber knob is usually ablated, possibly as a result of steric hindrance by adaptor molecules. One limitation is that complexes of Ad vectors and adaptor molecules are nonuniform, and batch-to-batch difference of the vectors might occur.

Chemical modification by polymers

Chemical modification with polyethylene glycol (PEG; PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase the blood persistence of therapeutic peptides and proteins (Harris and Chess, 2003). Modification of Ad vectors with PEG, in which the activated PEG reacts preferentially with the ϵ-amino terminal of lysine residues on the capsid, including the hexon, fiber, and penton base, prolongs persistence in the blood and circumvents neutralization of the Ad vectors by antibodies (O'Riordan *et al.*, 1999; Romanczuk *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004) (Fig. 1C). Furthermore, PEGylated Ad vectors attenuate the ability of the vector to be taken up by antigen-presenting cells, thereby reducing inflammatory responses. Animals administered PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression on readministration of unmodified Ad vectors in the lung (O'Riordan *et al.*, 1999; Croyle *et al.*, 2001). However, the PEGylation of Ad vectors leads to loss of infectivity due to steric hindrance by PEG chains (O'Riordan *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). The extent of loss of infectivity and extension of blood retention half-time are dependent on the degree of PEG modification (Eto *et al.*, 2004). The efficiency of transduction of 34% modified PEGylated Ad vectors was approximately 200-fold lower than that of unmodified Ad (Eto *et al.*, 2004).

To overcome the decreased efficiency of infection of PEGylated Ad vectors, vectors containing functional molecules on the tip of PEG have been developed (Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). Lanciotti *et al.* reported targeted Ad vectors, using heterofunctional PEG and FGF-2 (Lanciotti *et al.*, 2003). The transduction of Ad/PEG/FGF2 is dependent on the FGF-2 receptor, and is independent of CAR. In