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## High gene expression of the mutant adenovirus vector, both *in vitro* and *in vivo*, with the insertion of integrin-targeting peptide into the fiber

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In the present study, a first-generation adenovirus (Ad) vector was modified with the RGD peptide inserted into the fiber. The insertion of an integrin-targeting sequence into the Ad vector notably enhanced the luciferase expression in the Coxsackie virus and Adenovirus Receptor-deficient A2058 and B16BL6 melanoma cells. The results of an *in vivo* study with tumor-bearing mice also showed that Ad-RGD-Luc had enhanced gene expression in many organs and in the B16BL6 tumor compared to that induced by a conventional Ad vector after intravenous injection.

Adenovirus (Ad) vectors are widely used as carriers for gene therapy, both *in vitro* and *in vivo* (Asaoka et al. 2000; Gao et al. 2003; Gao et al. 2004). Recombinant Ad vectors can produce large amounts of gene products in a variety of dividing and nondividing cells. It has been reported that the initial process of Ad infection involves at least two sequential steps.

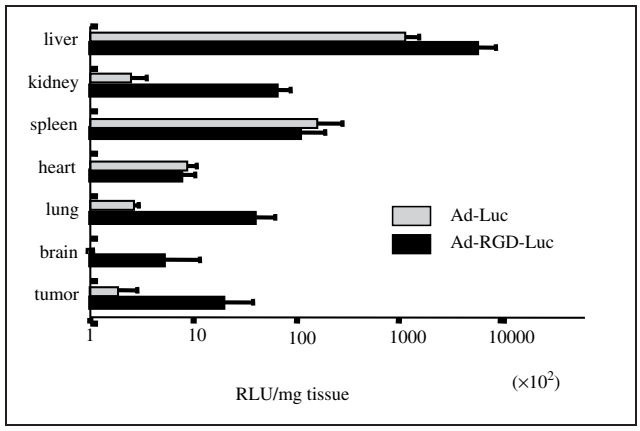


Fig. 2: Luciferase activity in organs after i.v. injection of Ad-Luc and Ad-RGD-Luc. The C57BL/6 mice were intradermally inoculated with $2 \times 10^5$ B16BL6 melanoma cells. After six days, $2 \times 10^9$ viral particles/mouse of Ad-Luc or Ad-RGD-Luc were injected into tail vein, respectively and the organs were harvested after 48 h. Then luciferase activity in organ homogenates was measured. Data are presented as the mean ± SD of relative light units (RLU)/mg tissue determined from four mice

The first step is the attachment of the virus to the cell surface, which occurs by binding of the fiber knob to the Coxsackie virus and Adenovirus Receptor (CAR) (Bergelson et al. 1997; Tomko et al. 1997). Following this, in the second step, the interaction between the RGD motif of the penton base with αv integrins, the secondary host-cell receptors, facilitates internalization by receptor-mediated endocytosis (Wickham et al. 1993; 1994). In other words, if the surface of host cells lack CAR, it is difficult to obtain an efficient gene transfer into those cells using a conventional Ad vector. For overcoming the low gene expression in CAR negative cells through Ad vectors, we developed a fiber-mutant Ad vector with an integrin-targeting RGD peptide by a simple *in vitro* method (Mizuguchi et al. 2001a). We anticipated that the fiber-mutant Ad system might target αv integrins during the first attachment to host cells. Therefore, this fiber-mutant system is an intriguing strategy for altering Ad tropism to enable efficient gene transduction into cells expressing little or no CAR. In the present study, we evaluated gene expression in A2058 human melanoma cells and B16BL6 mouse melanoma cells that are deficient in CAR and express ade-

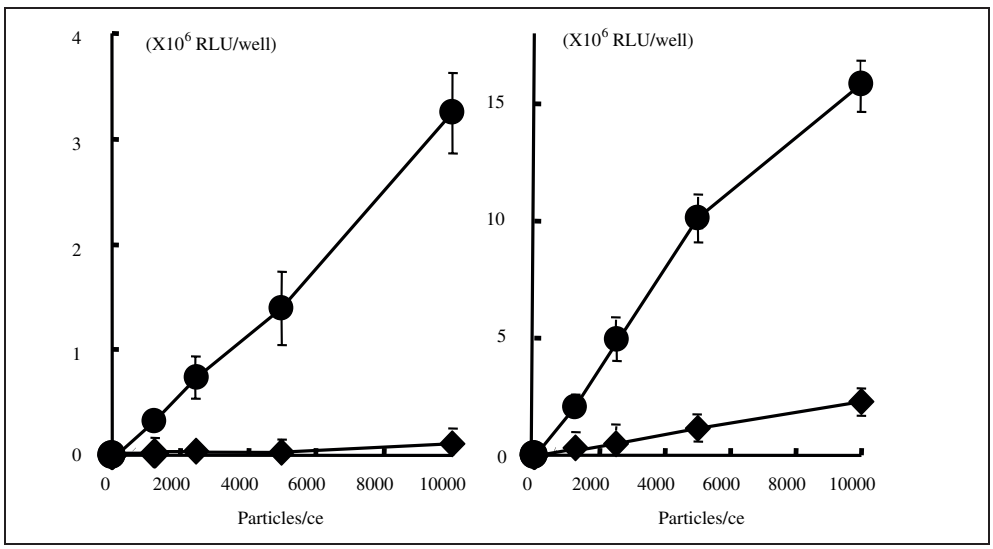


Fig. 1:
Luciferase expression of Ad-Luc or Ad-RGD-Luc infected A2058 and B16BL6 melanoma cells. A2058 cells (right) and B16BL6 cells (left) were transduced with Ad-Luc (◆) or Ad-RGD-Luc (●) respectively at the indicated viral particles/cell for 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured. Data are presented as the mean ± SD of relative light units (RLU)/well determined from the three experiments

quate levels of αv integrins, which was confirmed by RT-PCR (data not shown). As shown in Fig. 1, A2058 cells and B16BL6 cells infected using Ad-RGD carrying the luciferase gene induced significantly enhanced gene expression compared to that induced by the Ad-Luc.

Subsequently, the gene expression of Ad-RGD was evaluated *in vivo*. Ad-RGD or the conventional Ad encoding luciferase gene was injected intravenously in tumor-bearing mice and the luciferase activity in each organ was measured. After insertion of the RGD peptide into the HI loop of the fiber, the Ad-RGD showed a significantly increased luciferase activity compared to that induced by a conventional Ad vector in liver, lung, brain, and B16BL6 tumor, while it showed almost similar gene expression in spleen and heart (Fig. 2). Hence, the enhanced gene transfer in tissues, especially in brain and tumor makes this vector a useful and powerful carrier for efficient gene transduction and gene therapy.

## Experimental

### *1. Cell lines and animals*

B16BL6 mouse melanoma cells were maintained in Minimal Essential Medium (MEM) supplemented with 7.5% heat-inactivated Fetal Bovine Serum (FBS). The human embryonic kidney (HEK) 293 cells and A2058 human melanoma cells were cultured in DMEM supplemented with 10% FBS. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% $CO_2$. The C57BL/6 female mice (4 weeks old) were purchased from SLC Inc. (Shizuoka, Japan). All the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

### *2. Procedures*

#### *2.1. Construction of adenovirus vectors encoding RGD peptide in the fiber*

The replication-deficient adenovirus vectors used in this study were constructed from the adenovirus serotype 5 backbone with deletions of E1 and E3 and the expression cassette in the E1 region (Mizuguchi et al. 2001a). The integrin-targeting RGD sequence was inserted into the HI loop of the fiber knob using the two-step method. The fiber-mutant adenovirus vector, Ad-RGD-Luc carrying the luciferase gene under the control of the cytomegalovirus (CMV) promoter, was constructed by an improved *in vitro* ligation method as described previously (Mizuguchi and Kay 1998). A conventional vector encoding luciferase gene (Ad-Luc) was also developed. The Ad vectors were propagated in HEK 293 cells and purified by cesium chloride gradient ultracentrifugation, and their titer was determined by plaque-forming assay.

#### *2.2. Gene expression with Ad-RGD-Luc or conventional Ad-Luc in vitro*

The A2058 human melanoma cells and B16BL6 mouse melanoma cells were infected with Ad-Luc or Ad-RGD-Luc at 1250, 2500, 5000, and 10000 viral particles/cell for 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured using the Luciferase Assay System (Promega, USA) and Microlumat Plus LB96 (Perkin Elmer, USA) after the cells were lysed with the Luciferase Cell Culture Lysis (Promega, USA) according to the manufacturer's instruction.

#### *2.3. Gene expression with Ad-RGD-Luc or conventional Ad-Luc in vivo*

The C57BL/6 mice were intradermally inoculated with $2 \times 10^5$ B16BL6 melanoma cells. After 6 days, $2 \times 10^9$ viral particles/mouse of Ad-Luc or Ad-RGD-Luc were injected into the tail vein and the organs were harvested after 2 days. Subsequently, the luciferase activity in organ homogenates was measured using the method described in section 2.2.

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