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Fiber-modified adenovirus vectors mediate efficient gene transfer into undifferentiated and adipogenic-differentiated human mesenchymal stem cells

Hiroyuki Mizuguchi [a,b,*], Tomomi Sasaki [a], Kenji Kawabata [a], Fuminori Sakurai [a], Takao Hayakawa [c]

[a] *Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan*
[c] *Pharmaceuticals and Medical Devices Agency, Tokyo 100-0013, Japan*

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Abstract

Human mesenchymal stem cells (hMSCs) are considered a source of cells for regenerative medicine, and cell and gene therapy. Efficient gene transfer into hMSCs is essential for basic investigations into cellular differentiation and developmental biology, and for therapeutic applications in gene-modified regenerative medicine. In the present study, we optimized the transduction of hMSCs by means of fiber-modified adenovirus (Ad) vectors. Among the various types of Ad vectors tested, the polylysine modification of the C-terminal of the fiber knob most markedly improved the efficiency of hMSC transduction. At 300 vector particles per cell of polylysine-modified Ad vectors, more than 95% of the hMSCs expressed transgene. In this condition, polylysine-modified Ad vectors mediated 460-fold more transgene activity than the conventional Ad vectors. Ad vectors containing the Ad type 35 fiber or an Arg-Gly-Asp (RGD) peptide in the fiber knob mediated 130 or 16 times, respectively, the transgene activity mediated by the conventional Ad vectors. We also examined the efficiency of transduction into adipogenic-differentiated hMSCs. In this latter case, only Ad vectors containing the Ad type 35 fiber showed efficient gene expression. These results showed that fiber-modified Ad vectors could become a potent tool for basic research into, and the therapeutic application of, hMSCs and adipogenic-differentiated hMSCs.

Keywords: Adenovirus vector; Mesenchymal stem cells; Adipocytes; Gene therapy; Regenerative medicine

Bone marrow-derived mesenchymal stem cells (MSCs) have high proliferative capacity [1] and can differentiate into adipocytes, osteoblasts, and chondrocytes [2]. They can also differentiate into other types of cells such as nerve cells [3,4] and hepatocytes [5]. MSCs are considered vehicles for cell and gene therapy. As vehicles for cell therapy, MSCs are directly injected into the mesenchymal tissues, because these cells are progenitors of mesenchymal tissues. As vehicles for gene therapy, genetically modified MSCs are delivered systemically or injected directly to tissues of interest to express therapeutic proteins in the desired tissues. To generate genetically modified MSCs, it is essential to use a vector that efficiently mediates gene transfer into MSCs. An efficient gene transfer vector is also essential for basic research into MSCs, such as analyses of cellular differentiation and developmental biology.

Recombinant adenovirus (Ad) vectors continue to be the preferred vectors for gene therapy and the study of gene function. They are relatively easy to construct, can be produced at high titers, and have high transduction efficiencies. The efficiency of Ad vector-mediated transduction into human MSCs (hMSCs), however, is

* Corresponding author. Fax: +81 72 641 9816.
E-mail address: mizuguch@nibio.go.jp (H. Mizuguchi).

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quite low due to the scarcity of the primary receptor, called the coxsackievirus and adenovirus receptor (CAR) [6–9]. Therefore, hMSCs usually have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors [6,8,9]. Fiber-modified Ad vectors overcome this obstacle. We and other groups have developed several types of fiber-modified Ad vectors. One is constructed by the addition of foreign peptides to the HI loop or C-terminal of the fiber knob of an Ad vector [10–14]. Enhanced gene transfer has been reported, based on the use of mutant fiber proteins containing either an Arg-Gly-Asp (RGD) peptide [10–15] or a stretch of lysine residues (K7 (KKKKKKK) peptide) [10,14,15], which, respectively, target αv integrins or heparan sulfates on the cellular surface. Another type of fiber-modified Ad vector is made by removing fibers from one Ad serotype (Ad type 5) and replacing them with fibers derived from another—specifically, fibers that bind to receptor molecules other than CAR [16–20]. That is, fiber proteins derived from Ad belonging to the subgroup B, such as Ad type 3, 11, and 35, replace the Ad type 5 fiber. These fiber-modified Ad vectors infect cells via CD46, CD80, or CD86, which are recently identified cellular receptors of Ad belonging to subgroup B [21–25].

In the present study, we optimized the transduction to hMSCs by Ad vectors containing an RGD peptide in the HI loop of the fiber knob, Ad vectors containing a polylysine peptide in the C-terminal of the fiber knob, and Ad vectors containing a fiber protein derived from the Ad type 5 fiber tail, and the Ad type 35 fiber knob and shaft. The results showed that polylysine modification of the fiber knob greatly improved the efficiency of Ad vector-mediated transduction into hMSCs. We also report the efficient gene transfer into adipogenic-differentiated hMSCs by the Ad vectors containing Ad type 35 fiber.

Materials and methods

Ad vectors. Ad vectors expressing an *Escherichia coli* β-galactosidase (LacZ) were constructed by an improved in vitro ligation method [26,27]. The shuttle plasmid pHMCA-LacZ1 contains a CA promoter (a β-actin promoter/CMV enhancer with a β-actin intron) [this promoter/enhancer was kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan)] [28], the LacZ gene derived from pCMVβ (Clontech, Palo Alto, CA, USA), and a bovine growth hormone polyadenylation signal, all of which are flanked by I-CeuI and PI-SceI sites. I-CeuI/PI-SceI-digested pHMCA-LacZ1 was ligated with I-CeuI/PI-SceI-digested pAdHM4 [26], resulting in pAdHM4-CALacZ1. pAdHM15-RGD-CALacZ1, pAdHM41-K7-CALacZ1, and pAdHM34-CALacZ1 were constructed by the ligation of I-CeuI/PI-SceI-digested pHMCA-LacZ1 with I-CeuI/PI-SceI-digested pAdHM15-RGD [13], pAdHM41-K7 [14], and pAdHM34 [20], respectively. Viruses (Ad-CALacZ, AdRGD-CALacZ, AdK7-CALacZ, and AdF35-CALacZ) were generated with the transfection of PacI-digested pAdHM4-CALacZ1, pAdHM15-RGD-CALacZ1, pAdHM41-K7-CALacZ1, and pAdHM34-CALacZ1, respectively, into 293 cells per virus with SuperFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each virus was purified by $CsCl_2$ step gradient ultra-centrifugation followed by $CsCl_2$ linear gradient ultra-centrifugation. The virus particles and biological titer were determined spectrophotometrically by the method of Maizel et al. [29] and by using an Adeno-X Rapid Titer Kit (Clontech, Palo Alto, CA), respectively. The ratio of biological-to-particle titer was 1:22 for Ad-CALacZ, 1:26 for AdRGD-CALacZ, 1:32 for AdK7-CALacZ, and 1:21 for AdF35-CALacZ. The Ad vectors used in the present study were summarized in Table 1.

Cells. Bone marrow-derived hMSCs [purchased from Cambrex Bio Science Walkersville (Walkersville, MD)] were cultured with mesenchymal stem cell basal medium (MSCGM) (Cambrex Bio Science Walkersville) according to the manufacturer's instructions. hMSCs were used for experiments during passages two to four.

Adipocyte differentiation. The adipogenic-differentiated hMSCs were induced according to the manufacturer's instructions (Cambrex Bio Science Walkersville). In brief, hMSCs were seeded at a density of 2.1×10^4 cells/cm^2 and cultured with MSCGM for 10 days. The cells were then cultured with supplemented adipogenesis induction medium (Cambrex Bio Science Walkersville) for 3 days followed by 3 days of culture in supplemented adipogenesis maintenance medium (Cambrex Bio Science Walkersville). After three cycles of induction and maintenance, the cells were cultured with supplemented adipogenesis maintenance medium for 7 days. The differentiation of hMSCs to adipocytes was monitored by measuring intracellular lipid accumulation using Oil red O staining. In brief, the cells were fixed for 2 h with 10% formaldehyde in isotonic phosphate buffer and then washed with distilled water. The cells were then stained with complete immersion in a working solution (0.3%) of Oil red O for 4 h. Excess dye was removed by exhaustive washing with water.

Adenovirus-mediated gene transduction in vitro. hMSCs (1.1×10^4 cells) were seeded into a 24-well dish and the next day the cells were treated with each Ad vector for 1.5 h. Then, the medium containing the vectors was removed and fresh medium (MSCGM) was added to the cells. At the indicated amount of time, LacZ activity in the cells was measured by both X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining or the luminescence assay using a luminescent β-galactosidase genetic reporter system II (Clontech).

Flow cytometry. To detect human CAR, the cells (5×10^5 cells) were labeled with mouse monoclonal antibody RmcB (anti-human CAR) (Upstate Biotechnology, Lake Placid, NY) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Pharmingen, San Diego, CA). To detect human CD46, the cells were labeled with FITC-conjugated anti-human CD46 antibody (Pharmingen). Flow cytometric analysis was performed by a FACSCalibur flow cytometer using CellQuest software (Becton–Dickinson, Tokyo, Japan).

Results and discussion

To develop suitable Ad vectors for hMSCs, various types of fiber-modified Ad vectors expressing LacZ,

Table 1
Adenovirus vectors used in the present study

Name	Fiber type	Promoter
Ad-CALacZ	Type 5 fiber	CA promoter
AdRGD-CALacZ	RGD peptide in the HI loop of the fiber knob	CA promoter
AdK7-CALacZ	Polylysine peptide in the C-terminal of the fiber knob	CA promoter
AdF35-CALacZ	Chimeric type 5 fiber tail and type 35 fiber knob and shaft	CA promoter

CA promoter: β-actin promoter/CMV enhancer with β-actin intron.

which exhibit different tropisms than the conventional Ad vectors, were constructed (Table 1). The CA promoter, which is a hybrid promoter consisting of a β-actin promoter/CMV enhancer with a β-actin intron, was used in the following experiment because this promoter mediates ubiquitous and strong transgene expression [28,30]. Ad-CALacZ contains the wild-type fiber, AdRGD-CALacZ contains an RGD peptide motif in the HI loop of the fiber knob, AdK7-CALacZ contains a polylysine peptide in the C-terminal of the fiber knob, and AdF35-CALacZ contains a fiber protein derived from the Ad type 5 fiber tail and the Ad type 35 fiber knob and shaft. hMSCs were transduced with 100, 300, 1000, or 3000 vector particles (VP)/cell of each vec-

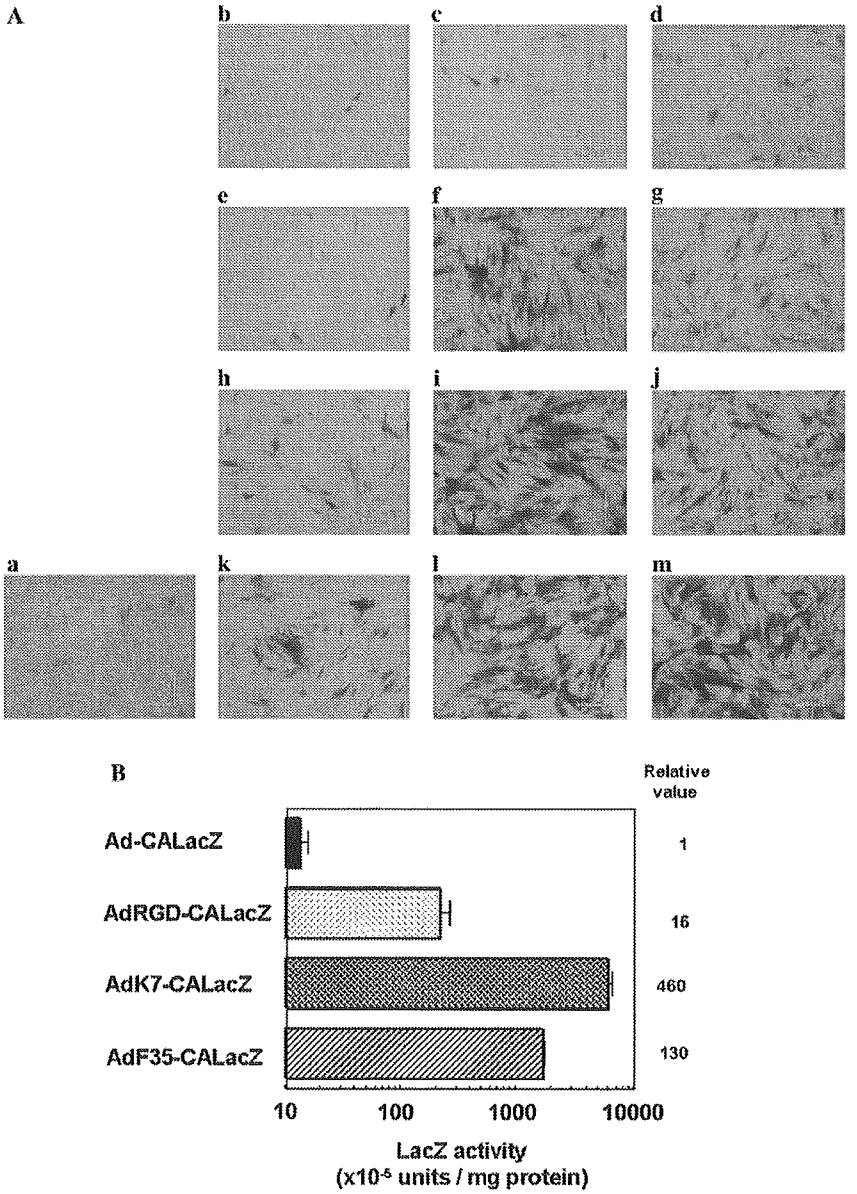

Fig. 1. LacZ expression of hMSCs transduced with various types of fiber-modified Ad vectors. (A) X-gal staining of hMSCs transduced with various types of fiber-modified Ad vectors. hMSCs were transduced with several Ad vectors, each containing one of the following: the wild-type fiber, RGD peptide in the HI loop of the fiber knob, polylysine peptide in the C-terminal of the fiber knob, or Ad type 35 fiber (Ad-CALacZ, AdRGD-CALacZ, AdK7-CALacZ, or AdF35-CALacZ, respectively) at 100, 300, 1000, or 3000 VP/cell for 1.5 h. Then, the medium containing the Ad vectors was removed and fresh medium (MSCGM) was added to the cells. X-gal staining was performed 48 h later. Note that 100% of the hMSCs were X-gal positive by transduction with AdK7-CALacZ (1000 and 3000 VP/cell) and AdF35-CALacZ (3000 VP/cell), and that the cells did not reach confluence. Data of X-gal staining are from one representative experiment of three performed. (a) Ad-CALacZ; (b, e, h, k) AdRGD-CALacZ; (c, f, i, l) AdK7-CALacZ; (d, g, j, m) AdF35-CALacZ. (b, c, d) 100 VP/cell; (e, f, g) 300 VP/cell; (h, i, j) 1000 VP/cell; and (a, k, l, m) 3000 VP/cell. (B) LacZ enzymatic activity of hMSCs transduced with various types of fiber-modified Ad vectors. hMSCs were transduced with 300 VP/cell of Ad-CALacZ, AdRGD-CALacZ, AdK7-CALacZ, or AdF35-CALacZ, respectively, for 1.5 h. Then, the medium containing Ad vectors was removed and fresh medium (MSCGM) was added to the cells. LacZ expression in the cells was measured by a luminescence assay 48 h later. The data of a luminescence assay are expressed as means ± SD ($n = 4$).

tor for 1.5 h, and the LacZ activity was determined by both X-gal staining and the luminescence assay (Fig. 1). In the case of the conventional Ad vector (Ad-CALacZ), the percentage of X-gal-positive cells was low even at the vector concentration of 3000 VP/cell. In contrast, all types of fiber-modified Ad vectors improved transduction efficiency. AdK7-CALacZ was the most effective in transducing the LacZ genes. Direct counting of the percentage of X-gal positive cells suggested that all of the hMSCs were transduced by AdK7-CALacZ at 1000 VP/cell. AdF35-CALacZ also showed high transduction efficiency. AdRGD-CALacZ mediated higher levels of LacZ expression than Ad-CALacZ, but lower levels than AdK7-CALacZ and AdF35-CALacZ (Fig. 1A). A luminescence assay showed that AdK7-CALacZ, AdF35-CALacZ, and AdRGD-CALacZ mediated 460, 130, and 16 times, respectively, the LacZ activity was mediated by Ad-CALacZ (Fig. 1B). Similar results were obtained in hMSCs derived from other donors.

Cytotoxicity and the ability of the transduced hMSCs to differentiate into adipocyte were also examined in hMSCs treated with polylysine-modified Ad vectors. The cell numbers were counted 3 days after transduction. No cytotoxicity was observed in hMSC treated with 1000 VP/cell of AdK7-CALacZ, but a slight cytotoxicity was seen in the cells treated with 3000 VP/cell of AdK7-CALacZ (the cell number was approximately 80% of the non-treated cells) (data not shown). hMSCs transduced with 1000 or 3000 VP/cell of AdK7-CALacZ differentiated into adipocyte as efficiently as non-treated cells (data not shown). Thus, the Ad transduction of hMSCs did not result in any impairment of proliferative and differentiated functions under the condition of 1000 VP/cell.

Tsuda et al. [31] reported that transduction efficiency into rat bone marrow-derived MSCs with an Ad vector containing RGD peptide in the HI loop of the fiber knob was 12 times that of a vector containing the wild-type fiber. Olmsted-Davis et al. [7] reported that human bone marrow-derived MSCs transduced with Ad vectors containing Ad type 35 fiber had higher levels of transgene expression than those transduced with conventional Ad vectors. The results of the present study, which is the first systemic comparison of transduction efficiency using various types of fiber-modified Ad vectors, suggested that polylysine modification of the C-terminal of the fiber knob was the most effective for the transduction of hMSCs.

Next, we examined the time course of transgene expression in hMSCs transduced with the Ad vectors, because the differentiation of hMSCs usually requires long-term cultivation. hMSCs were seeded into a 24-well dish and transduced with 1000 VP/cell of AdK7-CALacZ for 1.5 h. Then, the medium containing the Ad vectors was removed and fresh medium (MSCGM) was added to the cells. The cells reached confluence after 3–4 days and then became contact-inhibited. The cells were cultured without any passages. At days 2, 5, 13, 20, and 33, the LacZ expression in the cells was determined by a luminescence assay (Fig. 2). The results showed that LacZ expression was stable for 33 days, suggesting that Ad vector-mediated gene expression lasts at least a month in the cultured hMSCs when the transduced cells were cultured with contact-inhibition.

To determine why the Ad vector containing the wild-type fiber transduced hMSCs inefficiently, we examined the expression of CAR and CD46 in hMSCs by flow cytometry. The results showed that hMSCs express little CAR but higher levels of CD46 (Fig. 3), reflecting the lower or higher efficiency of gene expression by Ad-CALacZ or AdF35-CALacZ, respectively.

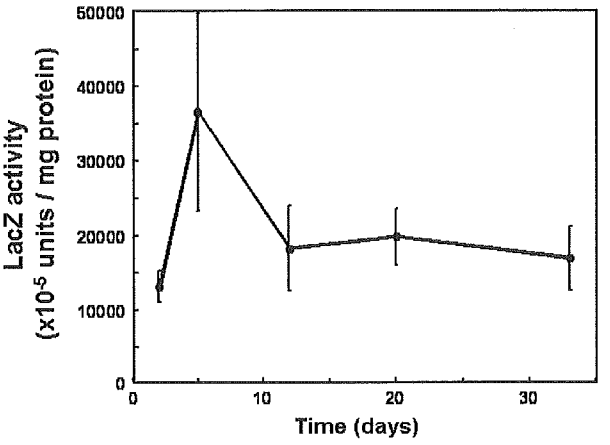

Fig. 2. Time course of LacZ expression in hMSCs transduced with AdK7-CALacZ. hMSCs were transduced with 300 VP/cell of AdK7-CALacZ for 1.5 h. Then, the medium containing Ad vectors was removed and fresh medium (MSCGM) was added to the cells. At the indicated times, LacZ expression in the cells was determined by a luminescence assay. The data of a luminescence assay are expressed as means ± SD ($n = 4$).

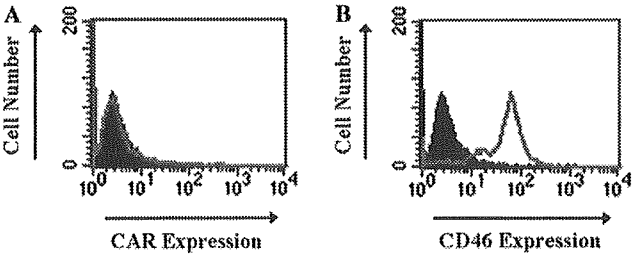

Fig. 3. Flow cytometric analysis of levels of CAR and CD46 expression in hMSCs. hMSCs were labeled with (A) mouse monoclonal antibody RmcB (anti-human CAR) and then FITC-conjugated goat anti-mouse IgG secondary antibody, or (B) FITC-conjugated anti-human CD46 antibody to detect human CAR or CD46 expression, respectively. As a negative control, the cells were incubated with an irrelevant antibody (shaded histogram). Flow cytometric analysis was performed by a FACSCalibur flow cytometer. Data shown are from one representative experiment of three performed.

We further examined the efficiency of transduction of adipogenic-differentiated hMSCs by fiber-modified Ad vectors. The adipocytes were induced from hMSCs according to the manufacturer's instructions (Cambrex Bio Science Walkersville), as described in Materials and methods. The differentiation of hMSCs to adipocytes was monitored by measuring the intracellular lipid accumulation using Oil red O staining (Figs. 4A and B). The transduction experiment was then carried out according to a protocol similar to that used for the hMSCs experiment. The cells were counted just before the experiment. The adipogenic-differentiated hMSCs were infected with each Ad vector at 300 VP/cell, and X-gal staining was performed 2 days later. As shown in Fig. 4C, Ad-CALacZ exhibited negligible LacZ expression in the cells with lipid vacuoles, i.e., the adipogenic-differentiated hMSCs. X-gal-positive cells were obtained in hMSCs without lipid vacuoles (non-adipogenic cells). AdRGD-CALacZ and AdK7-CALacZ led to an increase in LacZ-positive cells, but mediated LacZ expression in non-adipogenic cells (Figs. 4D and E). In sharp contrast, AdF35-CALacZ showed high LacZ expression in adipogenic-differentiated hMSCs only, and not in non-adipogenic cells (Fig. 4F). These results obtained further support from the flow cytometric analysis, in which adipogenic cells expressed high levels of CD46, but low levels of CAR (Fig. 5). The reason why AdF35-CALacZ mediated LacZ expression specifically in the adipogenic-differentiated hMSCs remains unclear, but these results may have been due to differences in the trafficking of the viral genome, relative CA promoter activity, and/or other factors in undifferentiated- vs. adipogenic-differentiated hMSCs. The hMSCs cultured with adipogenic medium expressed slightly more CAR than the uninduced hMSCs (Figs. 3 and 5); thus, the hMSCs cultured with adipogenic medium expressed more LacZ than the hMSCs cultured with normal medium (MSCGM) (Figs. 1 and 4). These results suggested that Ad vectors containing Ad type 35 fiber are of great utility for efficient transduction into adipogenic-differentiated hMSCs, and both RGD and polylysine modification of the fiber knob were found not to be effective for transduction into adipogenic-differentiated hMSCs.

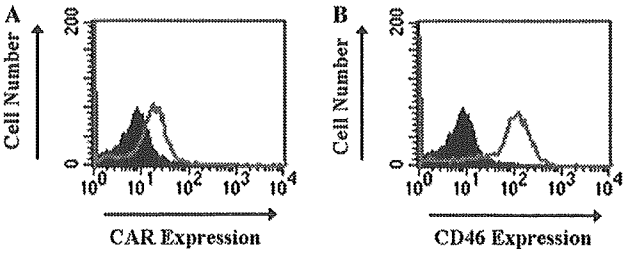

Fig. 5. Flow cytometric analysis of levels of CAR and CD46 expression of adipogenic-differentiated hMSCs. The adipogenic-differentiated hMSCs were labeled with (A) mouse monoclonal antibody RmcB (anti-human CAR) and then FITC-conjugated goat anti-mouse IgG secondary antibody, or (B) FITC-conjugated anti-human CD46 antibody to detect human CAR or CD46 expression, respectively. As a negative control, the cells were incubated with an irrelevant antibody (shaded histogram). Flow cytometric analysis was performed using a FACSCalibur flow cytometer. Data shown are from one representative experiment of three performed.

In summary, polylysine modification of the fiber knob and replacement of Ad type 5 fiber with Ad type 35 fiber in the Ad vectors exhibited the most efficient gene transfer into hMSCs and adipogenic-differentiated hMSCs, respectively. These types of fiber-modified Ad vectors are potentially useful for studies of gene function and also for the therapeutic application of hMSCs.

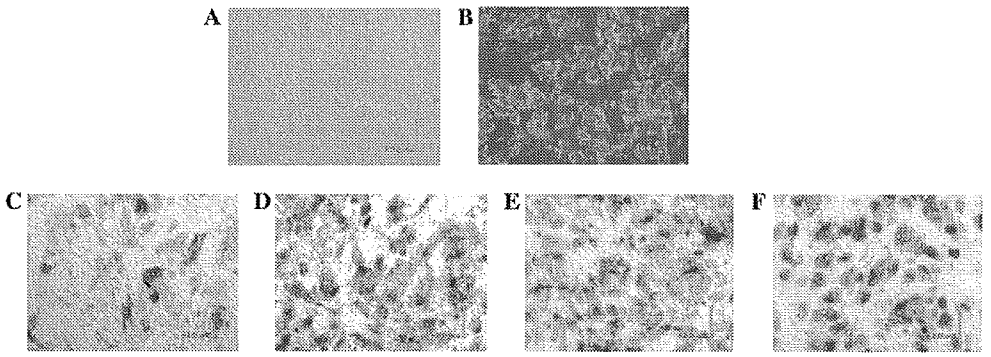

Fig. 4. X-gal staining of adipogenic-differentiated hMSCs transduced with various types of fiber-modified Ad vectors. Adipocytes were induced from hMSCs as described in Materials and methods. Intracellular lipid accumulation, which was used to mark adipocyte differentiation of uninduced (A) and induced hMSCs (B), was determined by Oil red O staining. The adipogenic-differentiated hMSCs were transduced with Ad vectors, each of which contained one of the following: the wild-type fiber (C), RGD peptide in the HI loop of the fiber knob (D), polylysine peptide in the C-terminal of the fiber knob (E), or Ad type 35 fiber (F) (Ad-CALacZ, AdRGD-CALacZ, AdK7-CALacZ, or AdF35-CALacZ, respectively) at 300 VP/cell for 1.5 h. Then, the medium containing Ad vectors was removed and fresh medium (supplemented adipogenesis maintenance medium) was added to the cells. X-gal staining was performed 48 h later. Data shown are from one representative experiment of three performed.

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Anti-tumor Responses Induced by Chemokine CCL19 Transfected into an Ovarian Carcinoma Model *via* Fiber-Mutant Adenovirus Vector

Jian-Qing GAO,[a,b] Toshiki SUGITA,[a] Naoko KANAGAWA,[a] Keisuke IIDA,[a] Naoki OKADA,[c] Hiroyuki MIZUGUCHI,[a,d] Takashi NAKAYAMA,[e] Takao HAYAKAWA,[f] Osamu YOSHIE,[e] Yasuo TSUTSUMI,[a,d] Tadanori MAYUMI,[a] and Shinsaku NAKAGAWA*,[a]

[a] *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan:* [b] *Department of Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University; 353 Yan-an Road, Hangzhou, Zhejiang 310031, P. R. China:* [c] *Department of Biopharmaceutics, Kyoto Pharmaceutical University; 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan:* [d] *National Institute of Biomedical Innovation; 7–6–8 Saito-Asagi, Ibaraki, Osaka 567–0085, Japan:* [e] *Department of Microbiology, Kinki University School of Medicine; Osaka-Sayama, Osaka 589–8511, Japan: and* [f] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.*

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Considerable attention has recently been paid to the application of chemokines to cancer immunotherapy because of their chemotactic affinity for a variety of immune cells and because several chemokines are strongly angiostatic. In the present study, the recombinant adenovirus vectors encoding chemokine CCL19 or XCL1 in an E1 cassette (AdRGD-mCCL19 and AdRGD-mXCL1) were developed. The constructed fiber-mutant adenovirus vector, which contained the integrin-targeting Arg-Gly-Asp (RGD) sequence in the fiber knob, notably enhanced the transfection efficiency to OV-HM ovarian carcinoma cells compared to that induced by conventional adenovirus vector. The results of an *in vitro* chemotaxis assay for chemokine-encoding vector demonstrated that both AdRGD-mCCL19 and AdRGD-mXCL1 could induce the migration of cells expressing specific chemokine receptors. Of the two chemokine-encoding vectors evaluated *in vivo*, AdRGD-mCCL19 showed significant tumor-suppressive activity in B6C3F1 mice *via* transduction into OV-HM cells, whereas XCL1 did not exhibit any notable anti-tumor effects, suggesting that CCL19 may be a candidate for cancer immunotherapy.

Key words chemokine; CCL19; XCL1; recombinant adenovirus vector; anti-tumor effect; OV-HM cell

Chemokines attract a variety of immune cells and function at inflammatory disease sites as well as lymphoid tissue.[1,2] Considering the eradication of tumor cells as a consequence of interaction with immune cells that have migrated and accumulated in tumor tissue, the usefulness of chemokines for cancer immunotherapy has received considerable attention.[3] By now, more than 40 chemokines have been well characterized, but only a few have been identified as candidates for cancer therapy either independently or with an adjuvant. Tumor-suppressive activity of several chemokines has been observed after transduction into a variety of experimental tumors.[4—7] Tumor cells that were transduced with the CC chemokine gene, CCL3, had reduced tumorgenicity and significantly increased infiltration of macrophages and neutrophils.[8] Another CC chemokine, CCL22, was also strongly chemoattractive to dendritic cells, NK cells and T cells, which resulted in tumor regression in a murine lung carcinoma model due to its efficient induction of anti-tumor immunity.[9] In the present study, we constructed the recombinant viral vector for efficient gene transfection and evaluated the CC family chemokine, EBI1-ligand chemokine (CCL19), and C family chemokine, lymphotactin (XCL1). CCL19 has been shown to chemoattract $CD4^+$, $CD8^+$ T cells and dendritic cells,[11,12] whereas XCL1 is chemotactic for T cells and NK cells but not for monocytes, neutrophils or dendritic cells.[13,14] We anticipated that if tumor cells could be genetically modified by an efficient gene transfer system *in vitro* to produce chemokines *in vivo*, the chemokines could induce accumulation of immune cells in the tumor. The *in vivo* interaction of T cells with the tumor cells should induce anti-tumor immunity, resulting in suppression of tumor growth.

In the present study, we used the adenovirus vector, which exhibits very high gene transduction efficiency.[15] Because a variety of tumor cells contain few Coxsackie adenovirus receptors (CAR),[16] we used a recombinant adenovirus vector with a fiber mutation containing the Arg-Gly-Asp (RGD) sequence in the fiber knob. This fiber-mutant vector possesses higher transduction and anti-tumor activities compared to conventional adenovirus vectors when used in cytokine-gene therapy against melanoma.[17,18] In the present study, ovarian carcinoma OV-HM cells were transfected with a chemokine-encoding recombinant vector, AdRGD-mCCL19 or AdRGD-mXCL1, and both the *in vitro* chemotactive activity and the *in vivo* tumor-suppressive response were investigated.

MATERIALS AND METHODS

Cell Lines and Animals OV-HM ovarian carcinoma cell line[19] were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Japan) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. A549 human lung carcinoma cells and human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. Murine pre-B lymphoma L1.2 cells and their stable transfectants, L1.2/mCCR7 and L1.2/mXCR cells, which expressing specific receptor for CCL19 and XCL1, respectively, were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 2-ME (50 μM, Life Technologies). All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Female B6C3F1 mice were purchased from SLC Inc. (Hamamatsu, Japan) and used at 6—8 weeks of age. All of the experimental pro-

* To whom correspondence should be addressed. e-mail: nakagawa@phs.osaka-u.ac.jp

cedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Construction of Adenovirus Vectors The replication-deficient adenovirus vectors containing a fiber mutation, which were used in this study, were developed based on the adenovirus type 5 backbone with deletions of the E1 and E3 regions.[20] The RGD sequence was inserted into the HI loop of the fiber knob using a two-step method developed by Mizuguchi *et al.*[21] Murine chemokine genes derived from pT7T3D-Pac-mCCL19 and pExCell-mCXL1 were used as sources of cDNA. Recombinant adenovirus vectors with the RGD fiber mutation, AdRGD-mCCL19 and AdRGD-mXCL1, carrying the chemokine cDNA under the control of the cytomegalovirus (CMV) promoter, were constructed by an improved *in vitro* ligation method described previously.[20,22] The luciferase expressing adenovirus vectors with the RGD fiber mutation (AdRGD-luc), serving as a negative control, is identical to the AdRGD-mCCL19 and AdRGD-mXCL1 vectors and contains the luciferase gene in the expression cassette (Fig. 1). Conventional adenovirus vector expressing lusiferase (Ad-Luc) was also developed by Mizuguchi *et al.*[22] The adenovirus vectors were propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation. Virus particle (VP) was accomplished spectrophotometrically.[23] The titer (tissue culture infectious $dose_{50}$; $TCID_{50}$) was determined by plaque-forming assay using 293 cells.[24,25]

Gene Expression by AdRGD-Luc or Conventional Ad-Luc in OV-HM Ovarian Carcinoma Cells 2×10^3 of OV-HM cells in a 96-well plate were treated with Ad-Luc or AdRGD-Luc at 1250, 2500, 5000, and 10000 viral particles/cell for 1.5 h, respectively. Cells were washed with PBS and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured using the Luciferase Assay System (Promega, U.S.A.) and Microlumat Plus LB96 (Perkin Elmer, U.S.A.) after the cells were lysed with the Luciferase Cell Culture Lysis (Promega, U.S.A.) according to the manufacturer's instruction.

***In Vitro* Chemotaxis Assay** The AdRGD-Luc and indicated AdRGD-chemokine were tranfected into A549 cells for 2 h at a multiplicity of infection (MOI) of 50, and the cells were washed twice with PBS and cultured in media containing 10% FBS. The cells were subsequently washed after 24 h cultivation, and incubated with an assay medium (phenol red-free RPMI 1640 containing 0.5% bovine serum albumin and 20 μM HEPES, pH 7.4) for another 24 h. The resulting conditioned medium was collected, and its chemoattractant activity was measured by an *in vitro* chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemotaxicell-24; Kurabo, Osaka, Japan) using L1.2 transfectants expressing the specific receptor for chemokines. The culture supernatants of intact A549 cells, AdRGD-Luc-transfected A549 cells, and chemokine gene-transduced A549 cells were prepared. These samples and recombinant chemokines dissolved in the assay medium were added to a 24-well culture plate. Cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1) were suspended in the assay medium (1×10^6 cells) and placed in a Chemotaxicell-24 installed on each well. Likewise, parental L1.2 cells for these transfectants were prepared and added to the Chemotaxicell-24. Cell migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The cells that migrated to the lower well were lysed and quantitated using a PicoGreen dsDNA quantitation reagent (Invitrogen, Tokyo, Japan). The data are expressed as mean±S.E. of the triplicate results and the migration activity was expressed in terms of the percentage of the input cells. Recombinant chemokines (mouse: mCCL19 and mXCL1) corresponding to each specific receptor (CCR7 and XCR1) were purchased from DakoCytomation (Kyoto, Japan) and used as a positive control.

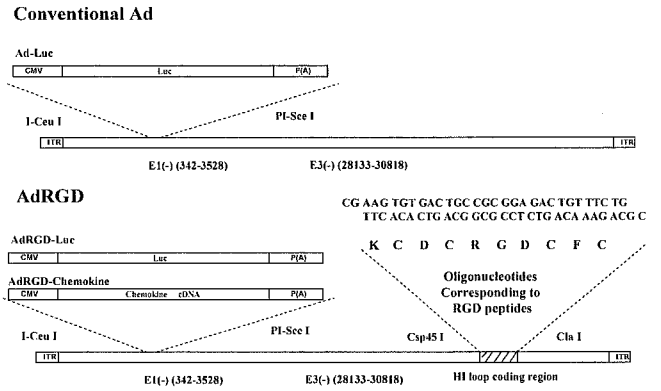

Fig. 1. Schematic Representation of Conventional Ad and AdRGD Used in This Study

Evaluation of Growth of OV-HM Cells Transfected with Chemokine-Encoding Adenovirus Vectors in Immunocompetent Mice OV-HM cells were transfected with AdRGD-mCCL19, AdRGD-mXCL1, or AdRGD-Luc as a control, at a MOI of 10 for 24 h. The cells were then harvested and washed with PBS three times and 1×10^6 cells were inoculated intradermally into the flank of B6C3F1 mice. An aliquot of the OV-HM cells infected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc at a MOI of 10 were cultured for an additional 48 h, and cell viability was examined by MTT assay. For *in vivo* evaluation of OV-HM cell growth, tumor volume was calculated by measuring the length and width of the tumor, twice a week. The mice were euthanized when one of the two measurements was greater than 15 mm.

RESULTS

OV-HM Tumor Cells Transfected with Fiber-Mutant Adenovirus Vector Induced Higher Gene Expression Than That Induced by Conventional Vector To evaluate the gene transfection efficiency of the fiber-mutant adenovirus vector developed for this study, OV-HM cells were transfected with conventional adenovirus vector or fiber-mutant adenovirus vector at indicated particles/cell and luciferase activity was measured. The results shown in Fig. 2 demonstrated that luciferase gene expression induced by fiber-mutant vector was much higher than that induced by conventional adenovirus vector. For example, at 10000 VP/cell transfection, 16-fold greater gene expression was obtained in response to fiber-mutant vector than to Ad-Luc. This demonstrated that the insertion of the RGD peptide into the viral fiber enhanced transfection efficiency to OV-HM cells *via* the adenovirus vector.

Expression of Murine CCL19 and XCL1 by Transfection with Chemokine-Encoding Adenovirus Vector To

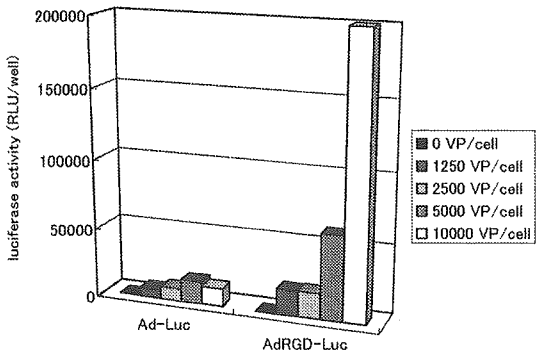

Fig. 2. Luciferase Expression by Ad-Luc or AdRGD-Luc Transfected OV-HM Cells

2×10^3 OV-HM cells were inoculated in a 96-well plate for 12 h, and were transfected with Ad-Luc (left) or AdRGD-Luc (right), respectively, at the indicated viral particles/cell for 1.5 h. The cells were then washed and incubated for another 48 h. After incubation, cells were collected and luciferase activity was measured. Data are presented as mean±S.E. of relative light units (RLU)/well determined from three experiments.

Table 1. Specific Chemoattractant Activity *in Vitro* Induced by Transfection of AdRGD-mCCL19 or AdRGD-mXCL1 into A549 Cells

	L1.2 % of input cells (mean±S.E.)	L1.2/XCR1 % of input cells (mean±S.E.)
Medium	0.2±0.0	0.1±0.0
10 nM mXCL1	0.3±0.0	9.2±0.8
A549	1.2±0.1	1.6±0.1
Luc/A549	1.5±0.0	2.0±0.2
mXCL1/A549	3.7±0.3	11.6±0.7

	L1.2 % of input cells (mean±S.E.)	L1.2/CCR7 % of input cells (mean±S.E.)
Medium	0.2±0.0	0.7±0.1
10 nM mCCL19	0.2±0.0	16.3±1.2
A549	1.2±0.1	2.0±0.1
Luc/A549	1.5±0.0	2.2±0.2
mCCL19/A549	2.5±0.1	8.2±0.5

Chemotaxis assay was performed using L1.2 cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1).

verify that the CCL19 and XCL1 produced by AdRGD-mCCL19 and AdRGD-mXCL1, respectively, were biologically functional, A549 cells were infected with the vectors for 2 h, and the culture supernatants were harvested after an additional 48 h. In the present study, human lung carcinoma A549 cells were used instead of murine tumor cells because of the very strong background chemotactic activity in the culture supernatant of the latter.[16] Using an *in vitro* chemotaxis assay, we investigated whether A549 cells transfected with each chemokine gene-carrying AdRGD could secrete chemokine protein in its biologically active form into culture supernatants. As shown in Table 1, the culture supernatants of A549 cells transfected with AdRGD-mCCL19 (mCCL19/A549) or AdRGD-mXCL1 (mXCL1/A549) could induce greater migration of cells expressing the corresponding chemokine receptors than those from intact A549 cells or A549 cells transfected with AdRGD-Luc (Luc/A549). The migration of L1.2 cells was not observed in wells containing recombinant chemokines, and only low-level migration was observed in culture supernatants from intact A549, Luc/A549, mXCL1/A549, and mCCL19/A549. These results demonstrated that all AdRGDs could deliver their encoded chemokine gene to target cells, and that transfected cells could secrete the chemokine protein, which maintained its original chemoattractant activity.

Anti-tumor Effect *in Vivo* by Transfection of Chemokine CCL19 into OV-HM Cells *via* Fiber-Mutant Adenovirus Vector OV-HM ovarian carcinoma cells transfected with 10 MOI of AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc as the control vector, were intradermally inoculated into B6C3F1 immunocompetent mice to evaluate their effects on tumor growth *in vivo*. In the present study, 10 MOI of Ad vectors were chosen for transfection because that higher MOI induced the cytotoxicity of OV-HM cells (data not shown). As shown in Figs. 3A and B, the transfection of AdRGD-mCCL19 resulted in significant suppression of tumor growth, while that of AdRGD-mXCL1 did not show any difference from that with AdRGD-Luc. To exclude the possibility that the suppression of tumor cell growth by AdRGD-mCCL19 was due to the cytotoxicity of the adenovirus or chemokine, OV-HM cells transfected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc were cultured for 48 h, and cell viability was measured by the MTT assay. The *in vitro* growth of the cells infected with these vectors was essentially identical to that of control cells (Fig. 3C).

DISCUSSION

Cytokines or chemokines encoded by a viral vector are currently regarded as intriguing options for cancer gene immunotherapy. Adenovirus vector, which shows high gene transduction efficiency and which can infect both dividing and non-dividing cells, is widely used as a carrier for gene therapy. It has been reported that the initial process of adenovirus infection involves at least two sequential steps. 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).[26,27] 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 through receptor-mediated endocytosis.[28,29] In other words, if the host cell surface lacks CAR, efficient gene transfer using a conventional adenovirus vector is difficult. Unfortunately, some malignant cells, including ovarian carcinoma, exhibit a resistance to adenovirus-mediated gene transduction due to low CAR expression on their surface. To overcome the low gene expression levels in CAR negative cells by adenovirus vectors, we constructed a fiber-mutant Ad vector with an integrin-targeting RGD peptide by a simple *in vitro* method.[20] The results of gene transfection *in vitro* (Fig. 2) demonstrated that OV-HM transfected using AdRGD-Luc carrying the luciferase gene significantly induced gene expression compared to that induced by the conventional Ad-Luc, suggesting that the recombinant adenovirus vector is a better option for cancer gene therapy.

We also inserted the murine chemokine cDNA of the CC family chemokine, CCL19, and C family chemokine, XCL1, into the E1 cassette of this fiber-mutant adenovirus vector, and AdRGD-mCCL19 and AdRGD-mXCL1 were developed. The expression of chemokine mRNA was reported pre-

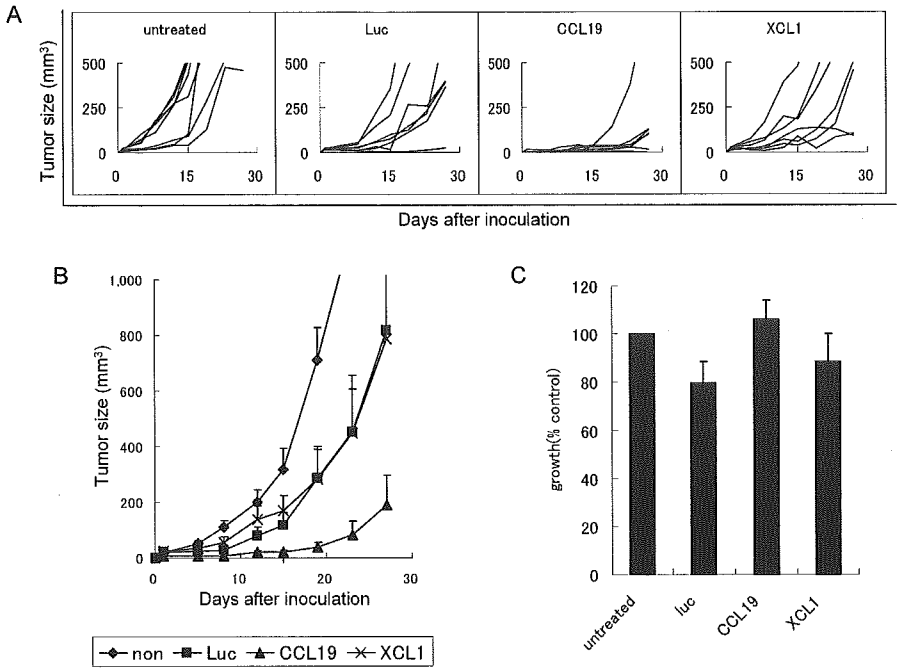

Fig. 3. Growth of OV-HM Tumor Cells in B6C3F1 Mice Transfected with Chemokine-Encoding Adenovirus Vectors

Mice were inoculated intradermally in the flank with 1×10^6 OV-HM cells (100 μl in RPMI 1640) at a MOI of 10 and with AdRGD-mCCL19 or AdRGD-mXCL1 for 24 h. Tumor volume was calculated after measuring the length and width of the tumor at indicated periods of time. Data are expressed as the mean±S.E. Intact OV-HM cells were used as control (untreated), and the OV-HM cells infected with AdRGD-Luc were inoculated into B6C3F1 mice for vector-control. Animals were euthanized when one of the two measured values were greater than 15 mm. At least six mice were used in each group. (A) Individual tumor size in each group and (B) average size in each group. (C) MTT assay results that evaluated the growth of chemokine-gene-transduced OV-HM cells *in vitro*. OV-HM cells were infected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc at a MOI of 10 for 24 h, and then cultured for 48 h. Cell viability was examined by MTT assay. Data are expressed as the means±S.E. of triplicate results. Each of the analyses were performed at least three times.

viously.[30] A chemotaxis assay of chemokine-encoding vectors was conducted *in vitro* to evaluate the biological activity of these vectors. The results demonstrated that the produced protein in the culture supernatants of cells infected with these vectors could efficiently cause migration of the specific receptor-expressing cells (Table 1).

The C family chemokine, XCL1, has been widely used for cancer immunotherapy, but in general, XCL1 by itself did not induce notable anti-tumor effects, even though it is a chemoattractant for both T cells and NK cells.[31] The CC chemokine, CCL19, reportedly induces T cell and dendritic cell migration and exhibits tumor-suppressive effects in several mouse malignant cell models.[32,33] Hillinger *et al.* reported that intratumoral injection of recombinant CCL19 led to significant systemic reduction in tumor volumes. CCL19-treated mice exhibited remarkably increased infiltration of $CD4^+$ and $CD8^+$ T cell subsets as well as dendritic cells at the tumor sites. These cell infiltrates were accompanied by increases in several cytokines and chemokines such as IFN-γ, CXCL9, CXCL10, GM-CSF, and IL-12.[34] We have also shown that CCL19 and XCL1 elicited anti-tumor response, to some extent, through transfection into B16BL6 melanoma cells. But our study, which used eight chemokines to evaluate the anti-tumor effects in three tumor cell types, suggests that the tumor-suppressive activity of chemokine gene immunotherapy is very complicated and is greatly influenced by the type of tumor and activation state of the host's immune system.[30] Moreover, as we previously reported,[10] transfection with the chemokine CCL27 induced tumor-suppressive effects, whereas another chemokine, CX_3CL1, did not show any notable anti-tumor activity. However, both of these chemokines induced the accumulation of T cells as well as NK cells at the tumor site. Our results indicated that the distribution of immune cells that have migrated to the tumor and the angiogenic or angiostatic activity may play an important role in the anti-tumor response.

Several groups have reported much stronger anti-tumor activity when using chemokines as adjuvants with other agents.[35—39] In the present study, CCL19 could not induce complete tumor regression, but merely inhibited its growth. On other hand, remarkable anti-tumor activity could be obtained when XCL1 was combined with cytokines or tranfected into dendritic cells.[40,41] A recent report showed that combination of both XCL1 and CXCL10 can enhance the efficiency of adoptive T cell therapy for EG7 tumor cells *via* accumulation of effector T cells in tumor tissue.[42] Many factors are likely to influence the tumor-suppressive effects of chemokines, but the relatively weak anti-tumor activity and long-term immuno-protective effects of chemokines may be mainly related to the activation level of migrating immune cells. In other words, not only the accumulation but also the activation of immune cells migrating into tumors is important in cancer immunotherapy using chemokines. Therefore, combination therapy using both chemokines and cytokines will increase the anti-tumor effects and improve cancer immunotherapy.

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Cancer Gene Therapy (2005) 00, 00–00

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Transcriptional targeting of RGD fiber-mutant adenovirus vectors can improve the safety of suicide gene therapy for murine melanoma

Yuka Okada,[1] Naoki Okada,[2] Hiroyuki Mizuguchi,[3] Takao Hayakawa,[4] Shinsaku Nakagawa,[5] and Tadanori Mayumi[5]

[1]Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; [2]Department of Biopharmaceutics, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan; [3]National Institute of Health Sciences, Osaka Branch, Fundamental Research Laboratories for Development of Medicine, 7-6-8 Asagi, Saito, Ibaraki, Osaka 567-0085, Japan; [4]National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; and [5]Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan.

Since RGD fiber-mutant adenovirus vector (AdRGD), which contains an αv-integrin tropism, is highly efficient in gene transduction to melanoma, the AdRGD-mediated herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) system is an attractive approach for melanoma treatment. However, the intratumoral injection of AdRGD causes limited transgene expression in healthy normal tissue, due to unwanted vector spread. Herein, we describe our attempt to overcome this limitation related to the safety of HSVtk/GCV treatment by using AdRGD carrying either melanoma-specific tyrosinase (Tyr) promoter or tumor-specific telomerase reverse transcriptase (TERT) promoter instead of universal cytomegalovirus promoter. Our *in vitro* study revealed that Tyr promoter-regulated AdRGD exhibited high transgene expression specificity for melanoma cells, and that TERT promoter-regulated AdRGD could induce efficient gene expression in tumor cells, but was relatively quiescent in normal cells. Anti-B16BL6 melanoma effects in mice injected intratumorally with AdRGD-Tyr/HSVtk or AdRGD-TERT/HSVtk, after which GCV was injected intraperitoneally for 10 days, were comparable to those in mice injected with AdRGD-CMV/HSVtk at 10 times less vector dosage. On the other hand, AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk did not induce severe adverse effects even when they were intravenously injected into mice at 10^9 plaque-forming units (PFU), whereas mice injected with AdRGD-CMV/HSVtk at 10^8 PFU exhibited body weight reduction and serum level increase of biochemical enzymes for hepatotoxicity. These results indicate that AdRGD combined with transcriptional regulation using Tyr or TERT promoter is a potentially useful and safe vector system for suicide gene therapy for melanoma.

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Keywords: suicide gene therapy; adenovirus vector; tissue-specific promoter; melanoma; hepatotoxicity

Currently, various strategies of gene therapy have been proposed for improving the cure rate of patients with melanoma,[1] whose prognosis is generally poor even after conventional treatment, such as surgery, chemotherapy, and radiotherapy.[2,3] Suicide gene therapy is a promising approach for melanoma, and the HSVtk/GCV system, which includes transduction of the herpes simplex virus thymidine kinase (HSVtk) gene followed by administration of the antiviral prodrug ganciclovir (GCV), has been the most widely studied method in preclinical and clinical settings.[4–10] Since HSVtk is an enzyme that converts nontoxic GCV to a highly toxic GCV-triphosphate, HSVtk-transduced cells render themselves sensitive to GCV, resulting in cell death. Moreover, an important advantage of the HSVtk/GCV system is the bystander effect, which confers cytotoxicity to untransduced cells adjacent to HSVtk-expressing cells by the transfer of GCV-triphosphate through gap junctions.[11–13]

We previously demonstrated that RGD fiber-mutant adenovirus vector (AdRGD), which contains an αv-integrin tropism due to an RGD peptide inserted into the HI loop of the fiber knob, was superior to conventional adenovirus vector (Ad) in gene transduction efficiency to melanoma both *in vitro* and *in vivo*.[14–16] In addition, Mizuguchi et al[17] reported that the intratumoral injection of AdRGD expressing the HSVtk gene under the

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Correspondence: Dr Naoki Okada, PhD, Department of Biopharmaceutics, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan.
E-mail: okada@mb.kyoto-phu.ac.jp

control of the cytomegalovirus (CMV) promoter, followed by intraperitoneal GCV administration, was approximately 25 times more effective at inducing tumor regression in established murine B16BL6 melanoma than injection of conventional Ad carrying the same expression cassette. These results suggest that AdRGD is a useful vector system for developing efficacious suicide gene therapy for melanoma because of its predominancy in gene transduction efficacy. However, we also found that AdRGD leaked from the injected tumor site into systemic circulation even if we carefully injected small volumes of AdRGD, and that a large fraction of the leaked AdRGD accumulated in the liver.[18] A major drawback of universal CMV promoter-based suicide gene therapy for cancer is the lack of selectivity for tumor cells in transgene expression accompanied by high probability for toxicity in normal healthy tissue.[19–21] Therefore, the ability to restrict gene expression to tumor cells is essential for assuring the safety of suicide gene therapy.

One possible approach to site-restricted suicide gene expression is the use of tissue-specific regulatory elements. For example, the tyrosinase (Tyr) promoter appears suitable for specific gene expression in melanoma cells. Tyr is a key enzyme in melanogenesis and is specifically expressed in pigmented cells including melanoma cells.[22,23] Likewise, telomerase reverse transcriptase (TERT) promoter would be a useful candidate for targeting transgene expression in cancer cells,[24–27] because TERT, the catalytic subunit of telomerase, is highly active in tumor cells but inactive in most normal cells.[28–30] Thus, in the present study, we constructed two new AdRGDs that express HSVtk gene under control of the Tyr or TERT promoter, and compared efficacy and toxicity of suicide gene therapy using these tumor-specific AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk with those using AdRGD-CMV/HSVtk.

Materials and methods

Cell lines and mice

Murine melanoma B16BL6 cells and human normal fibroblast WI-38 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Human melanoma A2058 cells, human alveolar adenocarcinoma A549 cells, and 293 cells, the helper cell line for AdRGD-expansion, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. Murine colon carcinoma Colon-26 cells were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Female C57BL/6 mice, aged 6–7 weeks, were purchased from SLC Inc. (Hamamatsu, Japan) and were held under specified pathogen-free conditions. Animal experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Cloning of the human TERT promoter

The human TERT promoter immediately upstream of the transcription start site (positions −378 to +77)[29] was amplified from human genomic DNA by polymerase chain reaction. The sequences of the specific primers were as follows: forward (5′-TGGCCCCTCCCTCGGGTTAC-3′) and reverse (5′-CGCGGGGGTGGCCGGGGC-3′). The amplified 455 bp fragment was subcloned into pGEM-3Zf(-) (Promega, Madison, WI), resulting in pGEM-hTERT2. The sequence was verified on a DNA sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA).

Vectors

Replication-deficient AdRGDs based on the adenovirus serotype 5 backbone with deletions of E1 and E3 regions were constructed by an improved *in vitro* ligation method using pAdHM15-RGD vector plasmid.[31–33] The AdRGD constructs are shown schematically in Figure 1. AdRGD-CMV/Luc[15] and AdRGD-CMV/HSVtk,[17] which express luciferase and HSVtk, respectively, under the control of the CMV promoter, were previously constructed by using pCMVL1[33] and pHM3-CMVtk[17] as shuttle plasmids. The human Tyr promoter with dual tandem melanocyte-specific (MS) enhancer was removed from pTyrex-2[34] (kindly provided by Dr DL Bartlett; Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) by restriction digest and inserted into pCMVL1 or pHM3-CMVtk instead of the human CMV immediate-early promoter and enhancer, resulting in

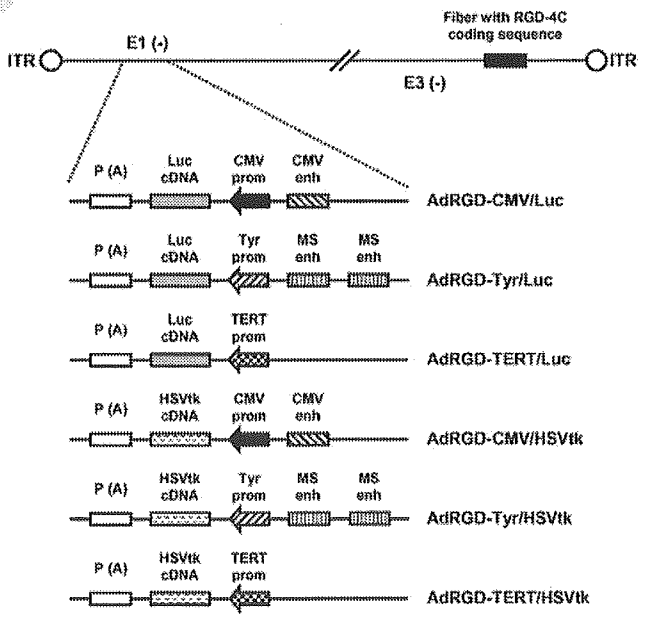

Figure 1 Schematic of AdRGDs used in this study. ITR, inverted terminal repeat; CMV prom, cytomegalovirus promoter; Tyr prom, tyrosinase promoter; MS enh, melanocyte-specific enhancer; TERT prom, telomerase reverse transcriptase promoter; Luc, firefly luciferase; HSVtk, herpes simplex virus thymidine kinase; P(A), polyadenylation signal.

pTyrL1 or pHM3-Tyrtk, respectively. Likewise, pTERTL1 or pHM3-TERTtk was constructed by replacement of CMV promoter in pCMVL1 or pHM3-CMVtk with the TERT promoter removed from pGEM-hTERT2 by restriction digest. Then, the I-*Ceu* I/PI-*Sce*I-digested fragment from each shuttle plasmid (pTyrL1, pTERTL1, pHM3-Tyrtk, or pHM3-TERTtk) was ligated to I-*Ceu*I/PI-*Sce*I-digested pAdHM15-RGD to create luciferase-expressing vector plasmids (pAdHM15-RGD-Tyr/Luc and pAdHM15-RGD-TERT/Luc) and HSVtk-expressing vector plasmids (pAdHM15-RGD-Tyr/HSVtk and pAdHM15-RGD-TERT/HSVtk), respectively. To generate the viral vector particle (AdRGD-Tyr/Luc, AdRGD-TERT/Luc, AdRGD-Tyr/HSVtk, and AdRGD-TERT/HSVtk), each vector plasmid was digested with *Pac*I to release the recombinant viral genome and transfected into 293 cells plated on a 100-mm dish with SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. All recombinant AdRGDs were propagated in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C. Titers (plaque-forming units, PFU) of infective AdRGD particles were evaluated by the end point dilution method using 293 cells.

In vitro *gene expression analysis*

B16BL6, A2058, Colon-26, A549, and WI-38 cells were seeded onto 24-well plates at 5×10^4 cells/well. On the following day, the cells were infected with AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc for 2 hours at 1 or 10 MOI (multiplicity of infection; PFU/cell) in 100 μl of FBS-free medium. Culture medium was added to each well after washing twice with phosphate-buffered saline (PBS). After 2 days, luciferase activity in the cells was determined by a luciferase assay system (Promega).

In vitro *cytotoxic assay*

B16BL6, Colon-26, and WI-38 cells were seeded onto 96-well plates at 4×10^3 cells/well. The next day, the cells were infected with AdRGD-CMV/HSVtk, AdRGD-Tyr/HSVtk, or AdRGD-TERT/HSVtk in 50 μl of FBS-free medium for 2 hours at MOI that did not induce cytopathic effects against the culture cells by transduction alone. The cells were then cultured in media containing GCV (Tanabe Pharmaceuticals, Osaka, Japan) at 0.08, 0.4, 2, or 10 μg/ml. After 4 days, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Dojindo Laboratories, Kumamoto, Japan) assay according to the method described by Mosmann.[35]

Tumor inoculation and intratumoral administration of vectors in animal experiments

B16BL6 cells were intradermally inoculated into the abdomen of C57BL/6 mice at 4×10^5 cells/mouse. After 6 days, each vector was injected into established tumors with diameters of 5–7 mm at various PFU in 50-μl PBS.

In vivo *suicide gene therapy model*

Established B16BL6 tumors were injected with AdRGD-CMV/HSVtk, AdRGD-Tyr/HSVtk, AdRGD-TERT/HSVtk, or AdRGD-CMV/Luc at 10^6, 10^7, or 10^8 PFU. The mice received daily injections of GCV (75 mg/kg) intraperitoneally for 10 days beginning the day after intratumoral injection with AdRGD. The major and minor axes of the tumor were measured using microcalipers, and the tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; $mm)^2 \times 0.5236$. The mice were euthanized when tumor volume was greater than 4000 mm^3. All survivors were euthanized on day 90 postintratumoral injection with AdRGD.

In vivo *gene expression analysis*

On day 2 after intratumoral injection of AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc at 10^6, 10^7, 10^8, or 10^9 PFU, the tumor, liver, spleen, kidney, heart, lung, and brain were removed, weighed, and homogenized in PBS containing 10 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride. Luciferase activity in the homogenates was determined by the luciferase assay system. Likewise, intact mice were intravenously injected with each vector at 10^7, 10^8, or 10^9 PFU, and then luciferase activity of the liver, spleen, kidney, heart, lung, and brain was measured 2 days later.

Systemic toxicity of three types of HSVtk-expressing AdRGDs

C57BL/6 mice were intravenously injected with AdRGD-CMV/HSVtk, AdRGD-Tyr/HSVtk, or AdRGD-TERT/HSVtk at 10^8 or 10^9 PFU. The mice received daily injections of GCV (75 mg/kg) intraperitoneally for 6 days beginning the day after intravenous AdRGD-injection. Body weight was measured every day, and the relative body weight was calculated by the following formula: (relative body weight) = (body weight after AdRGD treatment)/(body weight before AdRGD treatment). At 1 week after AdRGD injection, blood was collected from the tail vein of the mice, and the serum levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured using transaminase CII-testwako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's instructions.

Results

In vitro *transcriptional targeting of AdRGD carrying Tyr or TERT promoter*

By using melanoma cells, nonmelanoma tumor cells, and normal cells, we initially investigated whether AdRGD containing the luciferase gene driven by the Tyr or TERT promoter could target specific cells for gene expression (Fig 2). Melanoma cells (B16BL6 and A2058 cells) transduced with AdRGD-Tyr/Luc or AdRGD-TERT/

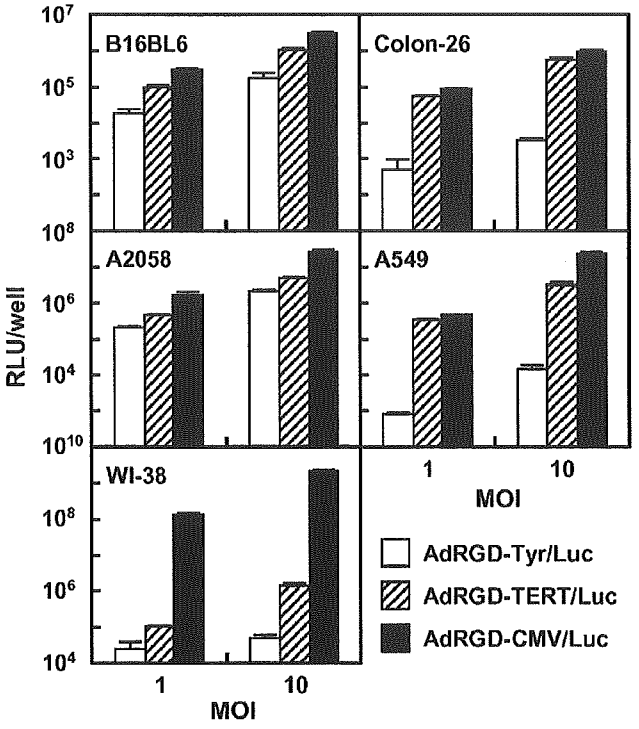

Figure 2 Promoter-regulated gene expression in melanoma cells and nonmelanoma cells transduced with luciferase by AdRGD. Murine melanoma B16BL6, human melanoma A2058, murine colon carcinoma Colon-26, human alveolar adenocarcinoma A549, and human normal fibroblast WI-38 cells were transduced with AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc at 1 or 10 MOI for 2 hours. After 2 days, luciferase activity was measured. Data represent the mean ± SD of relative light units (RLU)/well from four independent cultures.

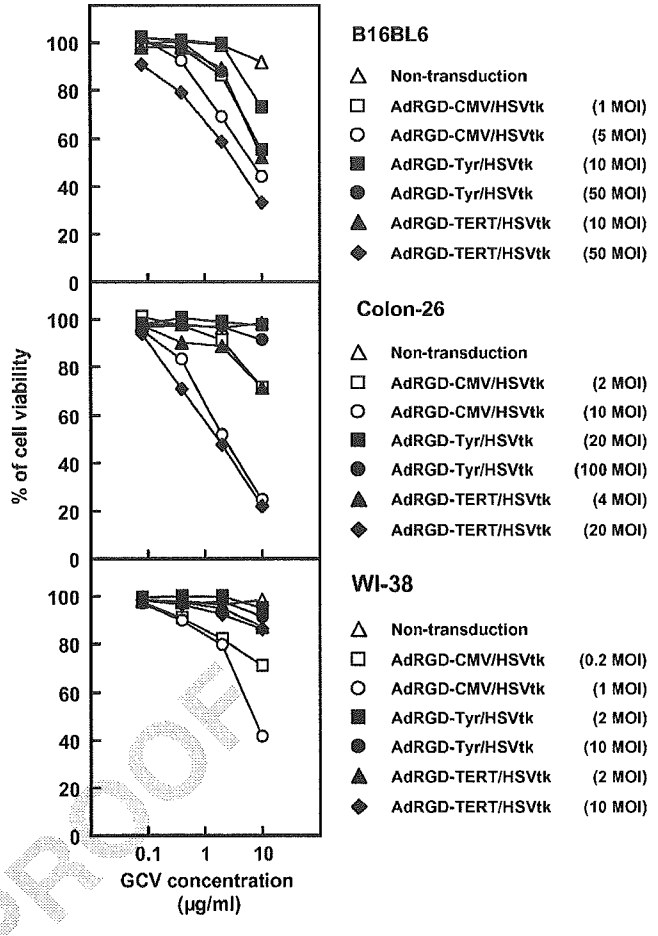

Figure 3 GCV sensitivity of cells transduced with different promoter-regulated HSVtk-expressing AdRGDs. B16BL6, Colon-26, and WI-38 cells were transduced with AdRGD-CMV/HSVtk (□, ○), AdRGD-Tyr/HSVtk (■, ●), or AdRGD-TERT/HSVtk (▲, ◆) at the indicated MOI for 2 hours, and these transduced cells and untransduced cells (△) were cultured with media containing GCV. Four days later, cell viability was measured by MTT assay. Data are expressed as a percent of viability of cells that were transduced with each AdRGD and cultured without GCV, and represent the mean of three independent experiments.

Luc exhibited satisfactory luciferase activities, and the transcriptional efficiency of Tyr and TERT promoters was only 8–18-fold and 3–5-fold lower than that of the CMV promoter, respectively. As expected, transduction with AdRGD-Tyr/Luc induced poor (2–3 log order lower level) transgene expression in nonmelanoma tumor cells (Colon-26 and A549 cells) in comparison with transduction with AdRGD-CMV/Luc, whereas AdRGD-TERT/Luc and AdRGD-CMV/Luc showed comparable luciferase activity, with a CMV promoter/TERT promoter ratio range of 1.4–7.1 in these cells. In addition, luciferase gene expression in a normal cell line (WI-38 cells) transduced with AdRGD-Tyr/Luc or AdRGD-TERT/Luc was 3–4 log orders lower than that in AdRGD-CMV/Luc-transduced cells.

Next, in order to confirm the specific cytotoxicity of the HSVtk suicide gene system driven by the Tyr and TERT promoters, we transduced B16BL6, Colon-26, and WI-38 cells with AdRGD-Tyr/HSVtk, AdRGD-TERT/HSVtk, or AdRGD-CMV/HSVtk and cultured these transduced cells in media containing GCV. Since the maximum vector dose that did not induce cytopathic effects in WI-38 cells was 10 MOI (data not shown), we used each vector at levels lower than 10 MOI for this normal cell line to profile cell death in response to GCV treatment. As shown in Figure 3, transduction with AdRGD-CMV/HSVtk decreased viability of all tested cells 4 days after transduction depending on GCV-concentration and vector dose. Reflecting the results shown in Figure 2, only B16BL6 cells exhibited a significant reduction in viability in response to AdRGD-Tyr/HSVtk and GCV treatment, and AdRGD-TERT/HSVtk rendered B16BL6 and Colon-26 cells sensitive to cytotoxicity due to GCV metabolism. Viability of normal WI-38 cells transduced with AdRGD-Tyr/HSVtk or AdRGD-TERT/HSVtk was not affected by GCV treatment. Taken together, these results demonstrated that AdRGD carrying the Tyr promoter could transcriptionally target melanoma cells for transgene expression, and that TERT promoter was highly active in tumor cells, but quiescent in normal cells.

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Antitumor efficacy of HSVtk/GCV system using different promoter-regulated AdRGDs

In order to compare the suppression of tumor growth between tumor-specific promoter-carrying AdRGD and universal promoter-carrying AdRGD in HSVtk/GCV therapy, we intratumorally injected AdRGD-Tyr/HSVtk, AdRGD-TERT/HSVtk, or AdRGD-CMV/HSVtk into established B16BL6 melanoma, and then administered GCV intraperitoneally into these mice for 10 days. Tumor volume change after AdRGD-injection and survival rate are summarized in Figure 4. Intratumoral injection with any HSVtk-expressing AdRGD could inhibit B16BL6 tumor growth in a vector dosage-dependent manner, and similar antitumor efficacy was observed between the AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk groups. In addition, 10^8 PFU of AdRGD-Tyr/HSVtk or AdRGD-TERT/HSVtk were needed to achieve comparable tumor regression and prolonged survival in mice to 10^7 PFU of AdRGD-CMV/HSVtk, that is, AdRGD-CMV/HSVtk was 10 times more effective.

Distribution of transgene expression in mice after intratumoral injection with different promoter-regulated AdRGDs

While intratumoral injection of AdRGD is an excellent method for attaining local elevation of transgene expression, prevention of AdRGD-leakage from the injected tumor into systemic circulation is very difficult.[18] Therefore, analysis of AdRGD biodistribution after the intratumoral injection is important for predicting and suppressing adverse effects of HSVtk/GCV treatment. We measured luciferase activity of B16BL6 tumors and six major organs (liver, spleen, kidney, heart, lung, and brain) in mice 2 days after intratumoral injection with AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc at 10^6–10^9 PFU (Table 1). Injection of any type of AdRGD increased luciferase activity in B16BL6 tumors in a vector dosage-dependent manner, and AdRGD-CMV/Luc-injected tumors exhibited 6–60 times higher luciferase activity than tumors injected with AdRGD-Tyr/Luc or AdRGD-TERT/Luc at the same PFU. This finding correlated closely with the difference in vector dosage between the three AdRGD-types, which was required for effective regression of B16BL6 tumor (Fig 4), indicating that the expression efficiency of HSVtk gene in tumor

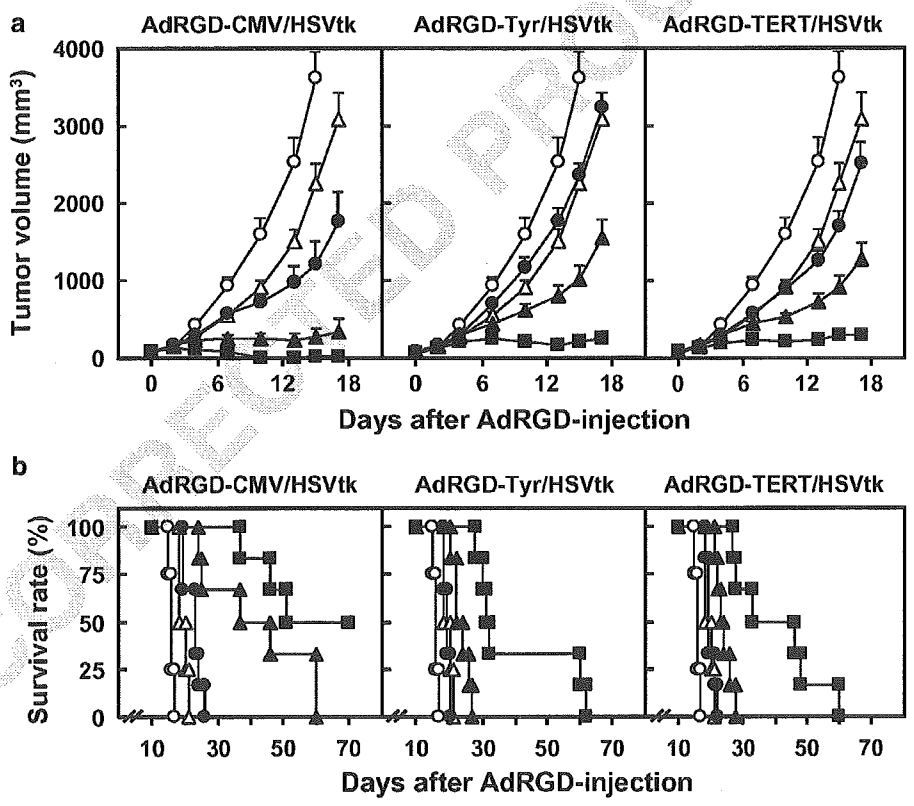

Figure 4 B16BL6 tumor growth inhibition and prolonged survival in response to the HSVtk/GCV system using different promoter-regulated HSVtk-expressing AdRGDs. Established B16BL6 tumors in C57BL/6 mice were injected with each HSVtk-expressing AdRGD at 10^6 (●), 10^7 (▲), or 10^8 (■) PFU in 50-μl PBS. Likewise, control groups were intratumorally injected with PBS (○) or AdRGD-CMV/Luc at 10^8 PFU (△). These mice were treated once daily with intraperitoneal injections of GCV for 10 days. (**a**) Tumor growth was monitored by calculating tumor volume. Each point represents the mean ± SE of 4–6 mice. (**b**) Mice containing tumors greater than 4000 mm^3 were euthanized. Percentage of survivors was calculated and plotted.

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Table 1 Luciferase activity in tumor and major organs from mice injected intratumorally with AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc

		RLU/tissue ($\times 10^{-7}$)						
Administered vector	PFU	Tumor	Liver	Spleen	Kidney	Heart	Lung	Brain
AdRGD-CMV/Luc	10^6	5.9±0.6	0.1±0.1	ND	ND	ND	ND	ND
	10^7	96±9	4.8±1.1	0.04±0.01	ND	ND	ND	ND
	10^8	1165±171	11±1.4	0.09±0.03	0.2±0.04	0.04±0.02	0.03±0.02	0.05±0.03
	10^9	15794±2131	73±27	0.4±0.06	0.9±0.3	0.2±0.07	0.3±0.06	1.0±0.4
AdRGD-Tyr/Luc	10^6	0.19±0.03	ND	ND	ND	ND	ND	ND
	10^7	1.9±0.3	ND	ND	ND	ND	ND	ND
	10^8	25±6.4	ND	ND	ND	ND	ND	ND
	10^9	231±55	ND	ND	ND	ND	ND	ND
AdRGD-TERT/Luc	10^6	1.0±0.07	ND	ND	ND	ND	ND	ND
	10^7	10±1.7	ND	ND	ND	ND	ND	ND
	10^8	82±19	0.2±0.1	ND	ND	ND	ND	ND
	10^9	269±35	0.8±0.2	ND	ND	ND	ND	ND

Established B16BL6 tumors in C57BL/6 mice were injected with each AdRGD at the indicated PFU in 50-μl PBS. After 2 days, the tumor, liver, spleen, kidney, heart, lung, and brain were removed and homogenized, and then luciferase activity in the homogenates was measured. All data represent the mean±SE of 5–6 mice. The mean background value of luciferase activity in each organ has been subtracted from the data. ND: luciferase activity was not detectable.

tissue is a critical factor for effectiveness of HSVtk/GCV treatment. In mice injected with AdRGD-CMV/Luc, luciferase activity was detected not only in the tumor but also in other organs, and more than 95% of total activity was observed in the liver. On the other hand, luciferase activity was not detectable in other organs from mice injected with AdRGD-Tyr/Luc at 10^6–10^9 PFU or AdRGD-TERT/Luc at 10^6 or 10^7 PFU. Although gene expression was observed in the liver from mice injected with AdRGD-TERT/Luc at more than 10^8 PFU, these luciferase activities were negligible and equivalent to activity in mice administered AdRGD-CMV/Luc at 10^6 PFU. Our data clearly revealed that AdRGD carrying Tyr or TERT promoter, which induced sufficient gene expression in melanoma tissue, could suppress transgene expression in other organs based on vector dissemination from injected tumor into systemic circulation.

AdRGD containing tumor-specific promoter reduces systemic toxicity of HSVtk/GCV system

To examine potential adverse effects of systemic leakage of the AdRGD-expressing suicide gene, mice were intravenously injected with AdRGD-CMV/HSVtk, AdRGD-Tyr/HSVtk, or AdRGD-TERT/HSVtk at 10^8 or 10^9 PFU, followed by intraperitoneal injection of GCV. Severe reduction of body weight was observed in the mice administered AdRGD-CMV/HSVtk at 10^8 PFU, whereas intravenous injection with AdRGD-Tyr/HSVtk or AdRGD-TERT/HSVtk did not induce considerable body weight change even at 10^9 PFU (Fig 5). When transgene expression levels were measured in six major organs from mice 2 days after intravenous injection with AdRGD-CMV/Luc at 10^7–10^9 PFU, the liver showed a marked increase in luciferase activity depending on vector dosage, and more than 99% of total luciferase activity

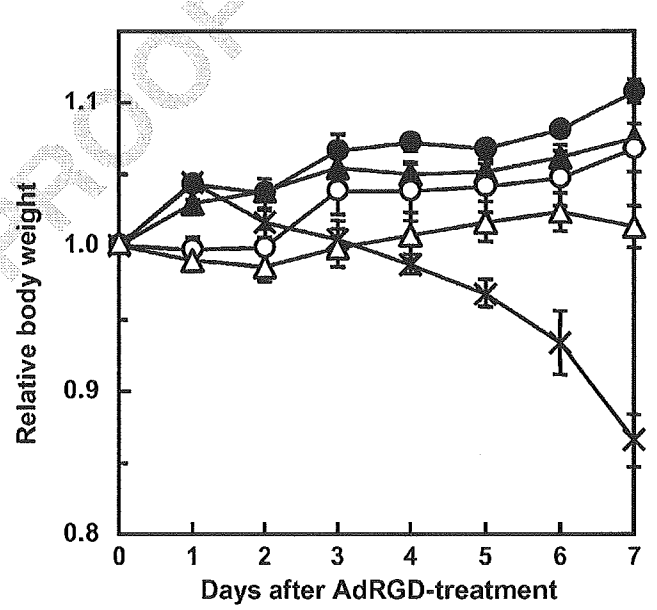

Figure 5 Body weight change upon an intravenous administration of different promoter-regulated HSVtk-expressing AdRGDs followed by GCV treatment. AdRGD-CMV/HSVtk (×; 10^8 PFU), AdRGD-Tyr/HSVtk (●; 10^8, or ; 10^9 PFU), or AdRGD-TERT/HSVtk (▲; 10^8, or △; 10^9 PFU) in 100-μl PBS were intravenously administered into C57BL/6 mice. Mice were treated with intraperitoneal injection of GCV for 6 days, and body weight was monitored every day. Relative body weight was calculated according to the formula described in the Materials and methods section. Each point represents the mean±SE of six mice.

was detected in the liver (Table 2). On the other hand, the liver from mice injected with AdRGD-Tyr/Luc or AdRGD-TERT/Luc at 10^9 PFU showed 1–4 log order lower luciferase activity than that from mice injected with

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Table 2 Luciferase activity in major organs from mice injected intravenously with AdRGD-CMV/Luc, AdRGD-Tyr/Luc, or AdRGD-TERT/Luc

		RLU/tissue ($\times 10^{-7}$)					
Administered vector	PFU	Liver	Spleen	Kidney	Heart	Lung	Brain
AdRGD-CMV/Luc	10^7	73±46	0.07±0.01	ND	0.01±0.01	0.02±0.02	ND
	10^8	971±279	1.3±0.2	0.5±0.3	0.1±0.02	0.2±0.04	0.1±0.02
	10^9	198867±88075	16±3.6	7.7±2.6	2.3±1	8.9±5.4	4.1 ±2.1
AdRGD-Tyr/Luc	10^7	ND	ND	ND	ND	ND	ND
	10^8	ND	ND	ND	ND	ND	ND
	10^9	0.08±0.07	ND	ND	ND	ND	ND
AdRGD-TERT/Luc	10^7	ND	ND	ND	ND	ND	ND
	10^8	0.06±0.08	0.05±0.01	ND	ND	ND	0.02±0.02
	10^9	24±7.0	0.4±0.1	0.04±0.03	ND	ND	0.04±0.04

C57BL/6 mice were intravenously injected with each AdRGD at the indicated PFU in 100-μl PBS. After 2 days, the liver, spleen, kidney, heart, lung, and brain were removed and homogenized, and then luciferase activity in the homogenates was measured. All data represent the mean±SE of six mice. The mean background value of luciferase activity in each organ has been subtracted from the data. ND: luciferase activity was not detectable.

Table 3 Serum activities of transaminases in mice after intravenous administration of different promoter-regulated HSVtk-expressing AdRGD followed by intraperitoneal injection of GCV for 6 days

Treatment	PFU	GOT (Karmen unit)	GPT (Karmen unit)
PBS	—	66±16	15±1
AdRGD-CMV/HSVtk	10^8	2262±724	855±174
AdRGD-Tyr/HSVtk	10^8	61±11	15±1
	10^9	115±40	20±6
AdRGD-TERT/HSVtk	10^8	77±17	14±4
	10^9	223±107	65±26

All data represent the mean±SD of six mice.

AdRGD-CMV/Luc at 10 times less vector dosage (10^8 PFU). These data strongly suggested that body weight reduction in response to HSVtk/GCV treatment was correlated with liver damage, which exhibited the highest levels of unfavorable transgene expression. Furthermore, blood was collected from mice that were treated with each HSVtk-expressing AdRGD and GCV, and then serum levels of GOT and GPT, enzymatic biomarkers of hepatotoxicity, were measured. As shown in Table 3, serum levels of GOT and GPT drastically increased in mice injected with AdRGD-CMV/HSVtk at 10^8 PFU. Although the histological examination of the liver did not show remarkable pathological change, the gallbladder of these mice was remarkably hypertrophied, and the serum showed a state of bilirubinemia (data not shown). In contrast, mice injected with AdRGD-Tyr/HSVtk or AdRGD-TERT/HSVtk at 10^8 or 10^9 PFU exhibited low GOT and GPT levels, less than one-tenth of those in mice administered AdRGD-CMV/HSVtk at 10^8 PFU. Therefore, AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk could reduce systemic adverse effects, mainly hepatotoxicity, of the HSVtk/GCV system by transcriptional targeting of the HSVtk gene to melanoma tissue.

Discussion

Suicide gene therapy using the HSVtk/GCV system is a potential cancer treatment in which therapeutic efficacy relies on the transduction efficiency of HSVtk gene into tumor. Among the currently available vector systems, Ad is frequently used in research and development of gene therapy due to highly efficient transduction in a wide variety of cell types and tissues regardless of the mitotic status of the cell. However, the efficiency of gene transfer using conventional Ad, which is derived from human adenovirus serotype 2 or 5, varies widely depending on the tissue origin of target cells. In particular, melanoma, which is an important target for gene therapy, requires high Ad dosage for sufficient gene expression because of the low expression or deficiency of the primary Ad-receptor, that is, the coxsackie-adenovirus receptor, on the cell surface.[14,36] In this regard, we previously demonstrated that AdRGD, which possesses αv-integrin tropism, was a potent vector system for gene transduction in melanoma, and that the intratumoral injection of AdRGD-expressing cytokine or HSVtk gene could more effectively induce tumor regression in established murine B16BL6 melanoma model than conventional Ad.[14–17] Although these results suggested that AdRGD could contribute to the establishment of efficacious suicide gene therapy for melanoma, gene expression had to be localized in tumor tissue when AdRGD encoding HSVtk gene was intratumorally injected in order to assure the safety of the HSVtk/GCV system. In fact, we found that about 1% of AdRGD that was carefully injected into B16BL6 tumor leaked from tumors into systemic circulation, although AdRGD could reduce systemic vector

dissemination by its superior gene transduction to melanoma as compared with conventional Ad.[18] In the present study, we attempted to construct a specialized AdRGD and optimized its applicability to the HSVtk/GCV treatment for melanoma by using Tyr (melanoma-specific) or TERT (tumor-specific) promoter.

In our *in vitro* transgene expression study using luciferase-expressing AdRGDs, the ratio of luciferase activities in human A2058 (melanoma) cells to those in human A549 (nonmelanoma) cells increased from 1–4 to 150–250 upon transduction with AdRGD-Tyr/Luc instead of AdRGD-CMV/Luc, whereas luciferase activities in A2058 cells transduced with either AdRGD-Tyr/Luc or AdRGD-CMV/Luc were comparable. Similarly, a drastic increase in gene expression specificity for melanoma was observed in murine cell lines, that is, the B16BL6/Colon-26 luciferase activity ratio was 36–55 for AdRGD-Tyr/Luc and 3.5 for AdRGD-CMV/Luc. McCart et al[37] also showed, by using conventional Ad-expressing luciferase under control of Tyr or CMV promoter, that the melanoma/nonmelanoma ratio was 6.3 for Tyr promoter and 0.14 for CMV promoter. These ratios were calculated by using the average luciferase expression levels in five human melanoma cell lines and six human nonmelanoma cell lines.[37] Since AdRGD exhibited more highly efficient gene transduction in melanoma lacking the coxsackie-adenovirus receptor than conventional Ad, we believed that melanoma-specificity of Tyr promoter could be improved by our AdRGD system. Likewise, the ratio of transgene expression levels in human tumor cell lines (A2058 and A549) to those in human normal cell line (WI-38) ranged from 2.3 to 4 for AdRGD-TERT/Luc and 0.003 to 0.01 for AdRGD-CMV/Luc. These values agreed with results of Gu et al,[24] who analyzed gene expression in five human tumor cell lines and two primary normal cells by using conventional Ad containing the CMV or TERT promoter. Taken together, our results revealed that AdRGD-Tyr/Luc transgene expression was selective for melanoma cells and that AdRGD-TERT/Luc could efficiently induce transgene expression in tumor cells, but not in normal cells.

AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk induced GCV sensitivity only in melanoma cells and tumor cells, respectively, whereas transduction with AdRGD-CMV/HSVtk followed by GCV treatment induced cytotoxicity in normal cells as well as melanoma and nonmelanoma tumor cells. Reflecting these encouraging *in vitro* results, intratumoral injection of AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk achieved not only potent antitumor efficacy in an established B16BL6 melanoma, but also drastic reduction in adverse systemic effects, mainly hepatotoxicity, which may be caused by HSVtk gene expression in organs distant from the tumor. Although AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk required administration at 10^8 PFU/tumor for attaining anti-B16BL6 tumor effects comparable to those from AdRGD-CMV/HSVtk injected at a 10-fold lower dose (10^7 PFU/tumor), intratumoral injection of AdRGD regulated with Tyr or TERT promoter induced little or no transgene expression in other organs even at 10^9 PFU. In contrast, undesirable transgene expression was detected in distant organs, mainly in the liver, upon intratumoral injection of AdRGD carrying the universal CMV promoter at a dosage capable of inducing effective tumor regression ($>10^7$ PFU). Importantly, AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk did not induce considerable adverse effects such as body weight reduction or increases in serum GOT/GPT activities, even when they were intravenously injected into mice at extremely high dosage, 10^8 or 10^9 PFU. Furthermore, luciferase expression in the liver from mice injected intravenously with AdRGD-Tyr/Luc or AdRGD-TERT/Luc at 10^9 PFU was lower than that in mice injected with AdRGD-CMV/Luc at 10^7 PFU. These results strongly suggest that AdRGD-Tyr/HSVtk and AdRGD-TERT/HSVtk dosage could be increased to potentiate antitumor efficacy without inducing adverse effects. Collectively, we demonstrated that through the addition of transcriptional targeting by using Tyr or TERT promoter, AdRGD, which possesses αv-integrin tropism resulting in highly efficient transduction, is a safer vector system for suicide gene therapy against melanoma.

Abbreviations

Ad, adenovirus vector; AdRGD, RGD fiber-mutant adenovirus vector; FBS, fetal bovine serum; GCV, ganciclovir; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HSVtk, herpes simplex virus thymidine kinase; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; PFU, plaque-forming unit; PBS, phosphate-buffered saline; RLU, relative light unit; TERT, telomerase reverse transcriptase; Tyr, tyrosinase.

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