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Transcriptionally Targeted *In Vivo* Gene Therapy for Carcinoembrionic Antigen-Producing Adenocarcinoma

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ABSTRACT

Inoperable adenocarcinoma in colon or lung shows resistance to conventional anti-cancer therapy. For these cancers, the feasibility of transcriptionally targeted killing of carcinoembryonic antigen (CEA)-producing adenocarcinoma cells was investigated.

Adenovirus vectors carrying a CEA promoter to express *E. coli lacZ* (AdCEALacZ) or herpes simplex thymidine kinase (AdCEATK) were made and their *in vitro* and *in vivo* tumoricidal effects on CEA-producing or non-producing colon and lung cancer cells were evaluated.

In vitro infection with AdCEALacZ showed significantly higher CEA promoter-driven *lacZ* expression in CEA-producing adenocarcinoma cells including VMRC-LCD and LoVo than in CEA-non-producing cells. AdCEATK-infected LoVo showed higher sensitivity to ganciclovir than control vector-infected LoVo or AdCEATK-infected HeLa both *in vitro* and in subcutaneously implanted tumors of nude mice. Moreover, total tumor elimination *in vivo* was achieved by either pre-infection of as few as 30% of cells comprising tumors or by direct *in vivo* injection of AdCEATK to pre-established LoVo tumors. In addition, CEA promoter-driven *lacZ* expression in LoVo cells was enhanced by the addition of interleukin-6 (IL-6) *in vitro*.

These results provide a rationale for CEA-promoter-driven, adenovirus-mediated gene therapy for CEA-producing adenocarcinomas in colon and lung with reduced toxicity to normal cells.

Key words: *Adenovirus-vector, HSV-TK, Interleukin-6, Cell-type-specific gene therapy*

Despite intensive efforts over three decades, there has been no meaningful improvement in the 5-year survival rate for patients with adenocarcinoma, which comprise about 97% of all colorectal carcinoma and 30% of all lung cancer cases, respectively[4,59,61]. In addition to chemo- and radioresistance, undetectable metastasis at diagnosis is a serious problem. Liver metastasis is the most common cause of death in patients with metastatic colorectal carcinoma[23,56]. In lung cancer, because clinically early-stage cases are often revealed to be more advanced than first thought by surgical-pathologic staging, the chances of successful curative surgery are not very high[37]. Since conventional therapies are far from satisfactory, the development of new therapeutic strategies, including gene therapy, for the treatment of adenocarcinoma is a priority.

Somatic gene therapy, treatment via genetic modification of the target cells, is being investigated extensively as a possible treatment for cancer[21,46,48]. A well-known strategy is the transfer of herpes simplex virus thymidine kinase (HSV-TK) gene followed by treatment with the anti-viral drug ganciclovir (GCV), where the nucleoside analogue is sufficiently phosphorylated to be incorporated into the DNA, resulting in cell death[2,5,35]. Plasmid vectors and virus vectors including retrovirus and adenovirus have been investigated as the vehicle of the HSV-TK gene[5,21,47,52,55]. Since the strategy is based on the toxicity of GCV phosphorylated by HSV-TK in the target cells, it requires highly efficient gene delivery to tumor tissue *in vivo*. Adenovirus vector has the advantage of being the only vector type available which is able to infect certain tissues with high efficiency[19,28,36,47,52,65]. However, adenovirus vectors used to transduce lethal or suicide genes to kill malignant cells would be toxic to normal tissues. In this regard, the high efficiency and broad spectrum of adenovirus vectors constitute a double-edged sword.

Possible solutions to this problem include the transduction of toxic genes to the target cells only or the expression of toxic genes in a target cell-specific fashion after random transduction. In the former context, strategies to deliver the genes in a target cell-specific manner have been investigated[13,50]. In the latter context, exploiting the transcriptional differences between normal and tumor cells with regulatory sequences of a tumor-associated marker gene including carcinoembryonic antigen (CEA) might be helpful in restricting the expression in target cells. The feasibility of putting a cell-type-specific promoter into various

vectors has been investigated. Osaki et al[38] and DiMaio et al[10] used the CEA promoter sequence -322 to +111 to express HSV-TK gene in CEA-producing lung cancer cell lines using a plasmid vector, and in pancreatic cancer cell lines using a retrovirus vector, respectively. With adenovirus vectors, Lan et al[26] used a site about 400 nucleotides upstream from the translation start site reported by Schrewe et al[51] to drive cytosine deaminase gene in gastric cancer cell lines *in vitro*. Tanaka et al[58] used the same sequence in adenovirus vectors to drive HSV-TK gene in gastric cancer cell lines and observed the inhibition of tumor growth *in vivo*. In the present study, with the CEA promoter sequence reported by Schrewe et al put into an adenovirus vector to drive HSV-TK gene, and using human cancer cell lines including CEA-producing colon and lung adenocarcinoma cell lines as targets, we show the tumor growth suppression by these vectors *in vitro* and *in vivo*, the *in vivo* bystander effect and the *in vitro* enhancement of cell-type-specific expression, and suggest that, by using appropriate promoters and lethal genes, a gene therapy for CEA-producing adenocarcinoma with reduced toxicity to normal tissues is possible.

MATERIAL AND METHODS

Adenovirus Vectors

The E1a$^-$, partial E1b$^-$ and partial E3$^-$ recombinant adenovirus vectors were engineered according to a technique previously described[28,36,65]. For the construction of adenovirus vectors containing CEA promoter, the *cis*-acting sequence -424 to -2 nucleotides upstream from the translational start site of CEA gene[51] was amplified from human genomic DNA with a pair of primers (sense: GATCGAATTCCTGGAGAGCATGGGGAGA; antisense: GATCCTCGAGTCTCTGCTGTCTGCTCTG). The amplified *cis*-acting sequence was inserted into the plasmid pXCJL.1 (a gift from Graham FL, McMaster University, Canada) along with HSV-TK (a gift from Evans RM, The Salk Institute for Biological Studies, Howard Hughes Medical Institute, La Jolla, CA) or *Escherichia coli* β-galactosidase (*lacZ*) gene. In a similar manner, the plasmid for the construction of the adenovirus vector containing cytomegalovirus (CMV) promoter to drive *lacZ* gene was prepared. The resulting plasmids were cotransfected with pJM17 (from Graham FL)[31] into 293 cells (ATCC CRL 1573, Rockville, MD) by the calcium phosphate precipitation method[17], and adenovirus vectors named AdCEALacZ, AdCEATK, and AdCMVLacZ were generated. The viral titers were determined by both plaque formation assay[17] and optical absorbance at 260 nm[44].

Cell Lines and Infection with Adenovirus Vectors

The CEA producing cell lines were the colon adenocarcinoma cell line LoVo and the lung adenocarcinoma cell lines A-549, VMRC-LCD, and VMRC-LCR. The CEA-non-producing cell lines were the cervical cancer cell line HeLa and the non-small cell lung cancer cell line ABC. All cell lines were obtained from the Japanese Cancer Research Resources Bank. They were maintained in minimal essential medium (MEM; GIBCO BRL, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS; MITSUBISHI KASEI corporation, Tokyo, Japan), 2 mM glutamine (GIBCO BRL), 100 units/ml penicillin, and 100 μM streptomycin (GIBCO BRL) at 37°C in a humidified incubator of 5% CO_2, unless otherwise specified. When the cells had grown to 70-80% confluence, they were infected with adenovirus vectors and were incubated in infection media (MEM containing 2 mM glutamine, 100 units/ml penicillin and 100 μM streptomycin) for 90 min at 37°C. As a control, parallel cultures were incubated in a similar manner without infection (sham infection).

Quantitative Analysis of *LacZ* Expression

To evaluate the ability of CEA promoter to express the transduced gene in a CEA-producing cell-type-specific manner, *lacZ* activity in AdCEALacZ-infected cells was analyzed. Quantitative analysis of *lacZ* expression was performed by measuring the conversion of *o*-nitrophenol-β-D-galactopyranoside to galactose and chromophore *o*-nitrophenol[27]. Briefly, 72 hr after infection with AdCEALacZ or AdCMVLacZ at a multiplicity of infection (moi) of 20 PFU/cell, the cells were dispersed and washed once with phosphate buffered saline (PBS; GIBCO BRL) and resuspended in a buffer containing 23 mM NaH_2PO_4, 77 mM Na_2PO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, and 40 mM β-mercaptoethanol (pH 7.3). Cell extract was prepared by 5 freeze-thaw cycles using liquid nitrogen and a 37°C water bath followed by centrifugation at 13,500 *g* for 15 min at 4°C to remove cellular debris. After adjusting the protein amount in each cuvet by measuring the protein concentration of each sample with a BCA protein assay reagent (Pierce, Rockford, IL), prewarmed *o*-nitrophenol-β-D-galactopyranoside was added to the cuvets (2.7 mM). After 30 min of incubation, Na_2CO_3 was added to each cuvet (0.3 M) to stop enzymatic conversion and sample absorbance was read at 420 nm. *LacZ* activity was calculated as follows: Unit=(380 × A420)/min.

GCV Sensitivity Assay *In Vitro*

AdCEATK-mediated *in vitro* sensitization of the cancer cell lines to GCV was evaluated. After infection with AdCEATK or AdCEALacZ (control vector) at moi 10, the cells were washed with PBS twice, resuspended in phenol red-free MEM (GIBCO BRL) containing 10% FCS and seeded

into 96 well-plates (Corning, New York, NY) at 6000 cells/well. After a 48 hour-incubation period, GCV (Syntex, Palo Alto, CA) was added to each well at various concentrations (0, 0.2, 2, 20, 200, 2000 μM) and incubation continued for 5 days. The cell viability was quantified using a DO TITE cell counting kit (WAKO, Osaka, Japan), a colorimetric cell proliferation assay kit, according to the manufacturer's protocol. The percentage of cell survival was calculated from the ratio of the absorbance measured of AdCEALacZ or AdCEATK-infected cells as compared with the sham infected cells.

In Vivo Growth Suppression of Tumor Cells Implanted to Nude Mice After *In Vitro* Infection

Animal experiments were performed with the approval of the animal welfare committee in Hiroshima University. The *in vivo* growth suppression of subcutaneously implanted cancer cells which had been pre-infected was evaluated in nude mice. Sham infected cells and cells infected with AdCEATK or AdCEALacZ at moi 10 were implanted at different sites (4×10^6 cells/site) in identical 5 week-old female Balb-c/AnNCrj-*nu/nu* mice (Charles River, Yokohama, Japan) subcutaneously. Seventy-two hours after implantation, when the cells had developed into tumors of about 4 mm in diameter, GCV was given to the mice intraperitoneally at a dose of 150 mg/kg body weight for two consecutive days. Two perpendicular diameters were measured using calipers, and tumor volume was estimated as follows: $V = A \times B^2/2$ (A = the largest diameter; B = the smallest diameter measured on the largest section of the tumor), up to 5 weeks after implantation.

In Vivo Direct Injection of Adenovirus Vectors to Pre-Existing Tumors in Nude Mice

Next, the growth suppression of pre-existing tumors by direct *in vivo* administration of adenovirus vectors was evaluated. HeLa or LoVo cells (4×10^6 cells/50 μl/site) were implanted in 5 week-old female Balb-c/AnNCrj-*nu/nu* mice subcutaneously. Seventy-two hours after implantation, AdCEALacZ or AdCEATK was injected into the tumors (8×10^8 plaque forming unit [pfu]/50 μl/tumor). Fifty μl PBS was injected as a blank. After an additional 72 hr, GCV was given to the mice and tumor volumes were estimated in a similar manner to that described above.

Evaluation of *In Vivo* Bystander Effect

To estimate the percentage of cells which needed to be transduced with HSV-TK gene for the total elimination of the tumors, the *in vivo* bystander effect was evaluated by mixing uninfected and infected cells. Ninety minutes after infection with AdCEATK at moi 10, LoVo cells were washed with MEM containing 10% heat-inactivated FCS and mixed with sham-infected cells at varying ratios (infected cells:sham-infected cells = 0:10, 1:9, 3:7 and 9:1). Then the cells were implanted to Balb-c/AnNCrj-*nu/nu* mice and GCV administration and tumor volume estimation were done in a similar manner to that described above.

Enhancement of CEA Promoter-driven *LacZ* Expression by Interleukin-6 *In Vitro*

Enhancement of CEA promoter-driven *lacZ* expression was also performed. LoVo cells were pre-incubated in medium containing recombinant human interleukin-6 (IL-6; PEPRO TECH EC LTD, London, England) at 20 ng/ml for 5 days, washed with PBS, then infected with AdCEALacZ at moi 20 and further incubated for 72 hr. Quantitative analysis of *LacZ* expression was performed by Fluorescence-activated-cell-sorter (FACS)-Gal analysis[27]. The cells were trypsinized and resuspended in staining media (PBS containing 10 mM HEPES, pH7.3; 4% FCS) at 10^7 cells/ml. Fifty microliters of cell suspension was incubated at 37°C for 10 min and 50 μl of prewarmed (37°C) 2 mM fluorescein-di-β-D galactopyranoside (FDG; MOLECULAR PROBES INS, Oregon, USA) was added. One minute's incubation was terminated by adding 2 ml of ice-cold staining media and the cells were then subjected to FACS analysis.

Statistical Analysis

Statistical analysis was performed by Kruskal-Wallis test followed by Fisher's protected least significance procedure for the detection and evaluation of the differences between groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

Cell-Type Specific Expression of *LacZ* Gene Driven by CEA Promoter *In Vitro*

There was no difference in the CMV promoter-driven *lacZ* activity among the CEA-non-producing HeLa, CEA-producing VMRC-LCD, VMRC-LCR, and LoVo cells, which ranged between 1.2 to 1.6 units/min/mg protein (Fig. 1). In contrast, CEA promoter-driven *lacZ* activity was significantly higher in VMRC-LCD, VMRC-LCR, and LoVo cells than in HeLa cells ($p<0.01$). Regardless of the promoter, the *lacZ* activity was lower in the CEA-non-producing ABC cells than in the other cells tested. In CEA-producing A549, although CMV promoter-driven *lacZ* activity was significantly higher than in any other cell lines tested ($p<0.05$), the CEA-promoter-driven *lacZ* activity was not favorable. The ratio of CEA promoter-driven *lacZ* activity to CMV promoter-driven *lacZ* activity was approximately 46% in LoVo

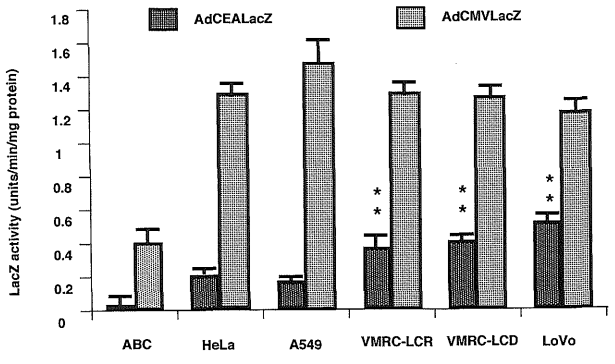

Fig. 1. Quantitative analysis of adenovirus-mediated *lacZ* expression *in vitro* driven by CEA or CMV promoter.
Seventy-two hours after infection with AdCEALacZ or AdCMVLacZ at moi 20, the cell extract was subjected to quantitative *lacZ* activity analysis by measuring the conversion of *o*-nitrophenol-*β*-D-galactopyranoside to galactose and chromophore *o*-nitrophenol, as detailed in the methods. Each column represents the mean of three separate experiments. T bars represent standard errors. **:Statistical significance compared to CEA promoter-driven *lacZ* expression both in HeLa and ABC ($p<0.01$) through Kruskal-Wallis test followed by Fisher's protected least significance difference procedure.

cells, four times and 30 times higher than the ratios in HeLa and ABC cells, respectively. Likewise, the ratio of the *lacZ* activity with CEA promoter to CMV promoter was approximately 32% in VMRC-LCD cells, and 27% in VMRCLCR cells, three times and 20 times higher than those in HeLa and ABC.

Cell Type-Specific Sensitization of Cancer Cell Lines to GCV *In Vitro* Driven by CEA Promoter

The *in vitro* GCV toxicity was markedly increased in AdCEATK-infected cells as compared with AdCEALacZ-infected cells in all the cell lines tested, whereas 20% of AdCEALacZ-infected VMRC-LCD cells were killed without addition of GCV (Fig. 2). When infected with AdCEATK at moi 10, 10 to 15% of HeLa or ABC cells were killed at 20 μM GCV concentration. In contrast, approximately 50% of the AdCEATK-infected LoVo and VMRC-LCD cells were killed in 20 μM GCV, which was lower than the recommended peak therapeutic plasma level of GCV in human, 39 μM [22].

In Vivo Growth Suppression of Pre-Infected Tumor Cells in a Cell-Type Specific Fashion

After subcutaneous implantation followed by

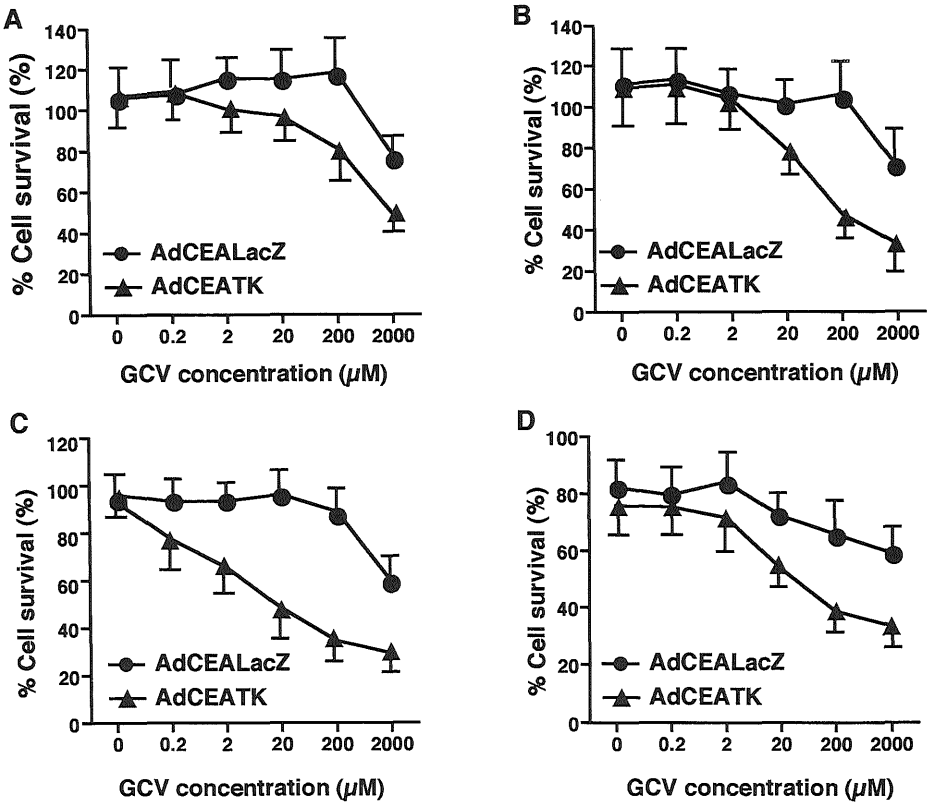

Fig. 2. AdCEATK-mediated sensitization to GCV *in vitro*.
Cells infected with AdCEATK or AdCEALacZ at moi 10 were seeded into 96 well-plates at 6000 cells/well. After a 48 hour-incubation, GCV was added to the wells at various concentrations. The cells were incubated for an additional 5 days and the viable cell number was evaluated and expressed as a percentage of cell number of sham infected cells. (A) ABC; (B) HeLa; (C) LoVo; (D) VMRC-LCD. Data indicate the mean values of 15 individual determinations. T bars represent standard errors.

GCV administration, the HeLa cells infected with AdCEATK developed into obvious tumors, though these were smaller in size than those of the sham-infected and control vector-infected cells. However, surprisingly, the LoVo cells infected with AdCEATK formed no tumors throughout the period of observation, whereas the sham-infected LoVo cells and the LoVo cells infected with control vector developed into apparent tumors in the same mice (Fig. 3). While there was no difference in tumor volume among the uninfected HeLa, control vector-infected HeLa cells, or AdCEATK-infected HeLa at 4 weeks after implantation (Fig. 4A), the AdCEATK-infected LoVo cells formed no tumors, and were significantly different at three and 4 weeks after implantation compared with the control vector-infected LoVo ($p<0.05$ at 3 weeks and $p<0.01$ at 4 weeks after implantation) and uninfected LoVo cells ($p<0.01$ at 3 and 4 weeks after implantation, Fig. 4B).

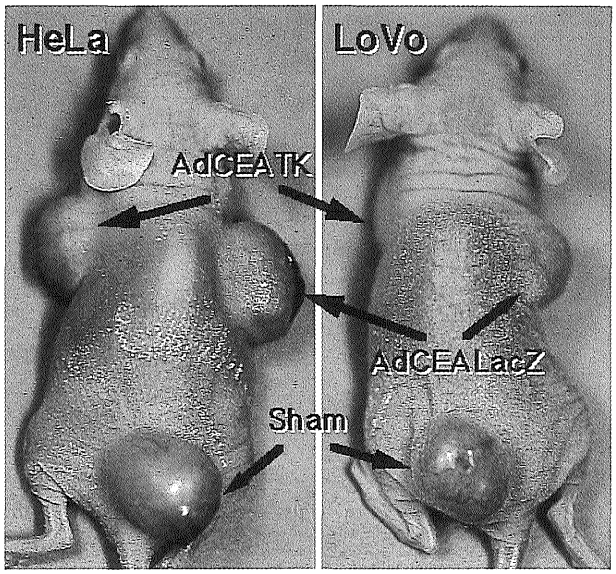

Fig. 3. *In vivo* cell type-specific growth suppression of pre-infected tumor cells in nude mice.
HeLa cells or LoVo cells infected with AdCEALacZ or AdCEATK at moi 10 were injected subcutaneously to different sites (4×10^6 cells/site) of 5 week-old female Balb-c/AnNCrj-*nu*/*nu* mice. After 72 hr, GCV was given to the mice intraperitoneally (150 mg/kg body weight) for 2 days. Shown are mice at 4 weeks after implantation of HeLa cells and LoVo cells.

Growth Suppression of Pre-Existing Tumors by Direct *In Vivo* Administration of Adenovirus Vectors

After direct intratumoral injection, there was no significant difference in the volume of the HeLa tumors regardless of the vectors injected throughout the period of observation (Fig. 5A). In contrast, the growth of the LoVo tumors was suppressed after AdCEATK injection compared with both the saline-injected tumors and AdCEALacZ-injected tumors throughout the period of observation (Fig. 5B), and the difference was significantly different at 3, 4, and 5 weeks after implantation for saline-injected tumors ($p<0.01$) and at 3 weeks for AdCEALacZ-injected tumors ($p<0.05$). Unlike the LoVo cells pre-infected with AdCEALacZ, the growth of LoVo tumors directly injected with AdCEALacZ was suppressed significantly compared with saline-injected tumors ($p<0.05$ at 3 weeks after implantation), which was probably due to the sensitivity of LoVo cells to high-dose

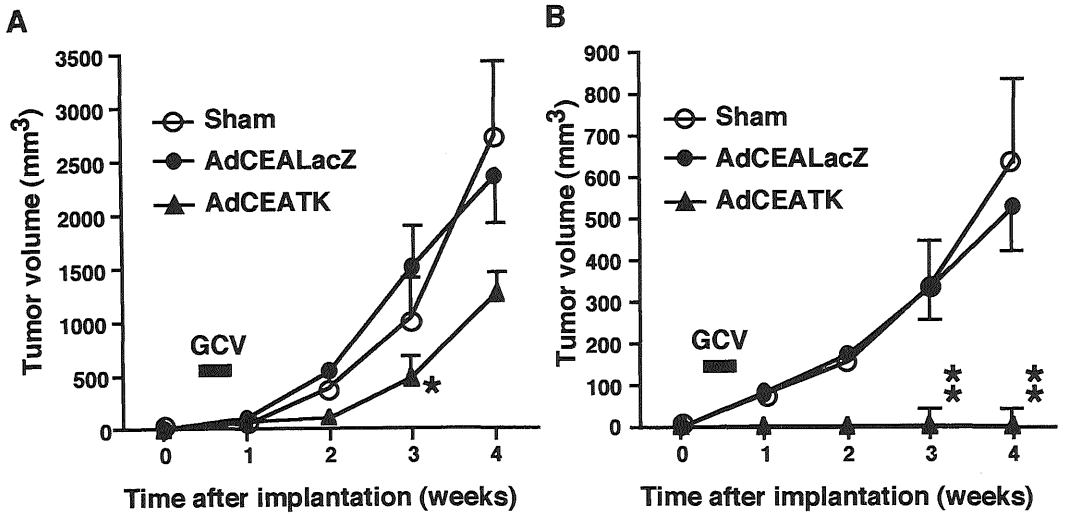

Fig. 4. Tumor volume as a function of time after implantation to nude mice.
After implantation of pre-infected cells to the nude mice followed by GCV administration, two perpendicular diameters were measured and tumor volumes were estimated as detailed in the methods. Data indicate mean values of tumor volumes of HeLa cells (A, n=4) and LoVo cells (B, n=5). T bars represent standard errors. *, **:Statistical significance to sham- infected tumors (*:$p<0.05$; **:$p<0.01$) through Kruskal-Wallis test followed by Fisher's protected least significance difference procedure.

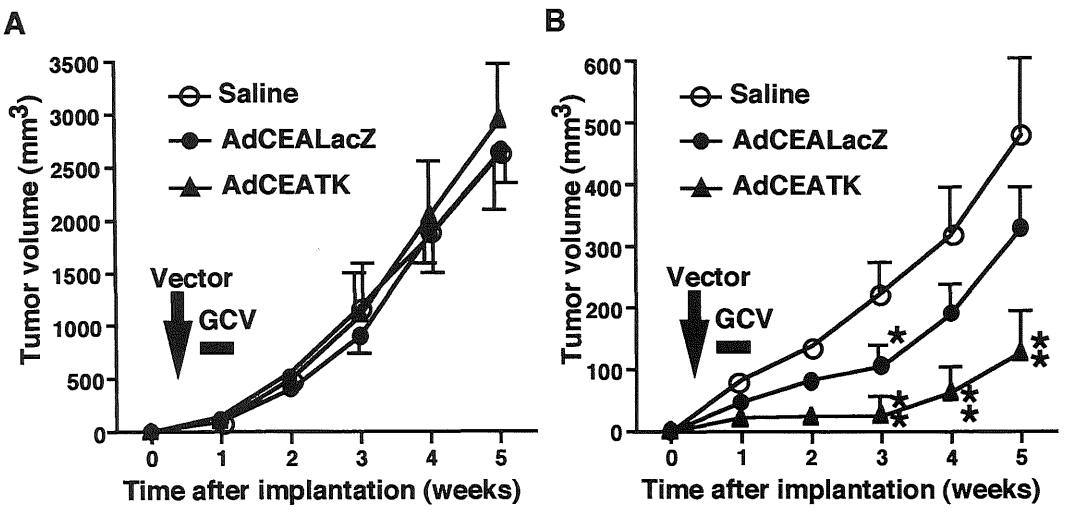

Fig. 5. Growth suppression of pre-existing tumors by direct intratumoral administration of adenovirus vectors *in vivo*.
Uninfected HeLa or uninfected LoVo cells were implanted into 5-week-old female Balb-c/AnNCrj-*nu*/*nu* mice subcutaneously (4×10^6 cells/site). Seventy two hours after implantation, AdCEALacZ or AdCEATK was injected into the tumors (8×10^8 pfu/tumor). PBS was injected as a buffer control. After an additional 72 hr, GCV was given to the mice and tumor volumes were estimated as detailed in the methods. Data indicate mean values of tumor volumes of HeLa cells (A, n=6) and LoVo cells (B, n=6). T bars represent standard errors. *, **:Statistical significance compared to saline-injected tumors (*:$p<0.05$; **:$p<0.01$) through Kruskal-Wallis test followed by Fisher's protected least significance difference procedure.

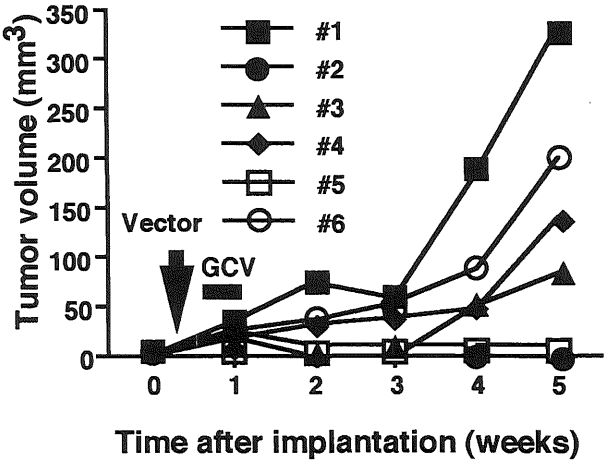

Fig. 6. The volume of each LoVo tumor as a function of time after AdCEATK intratumoral injection.
In two out of six mice, the tumor was totally eliminated by AdCEATK injection followed by GCV administration (#2 and #5). In mouse #3, regrowth was observed after the tumor had once disappeared.

adenovirus infection. In two out of six mice, the tumors were totally eliminated by AdCEATK injection followed by GCV administration (#2 and #5). In mouse #3, the tumor seemed to disappear, but regrowth of residual cells occurred (Fig. 6).

Suppression of Tumor Growth by *In Vivo* Bystander Effect

When 90% of the LoVo cells had been pre-infected with AdCEATK at moi 10, the cell mixture did not develop into an obvious tumor after subcutaneous implantation followed by GCV administration. In addition, when 30% of the LoVo cells had been pre-infected, the cell mixture developed into an obvious tumor in only one out of six mice. Further, even when as few as 10% of the cells had been pre-infected, the tumor growth of the mixed cells was significantly reduced compared with the tumors of the shaminfected cells ($p<0.05$ at 4 and 5 weeks after implantation, Fig.7).

In Vitro Enhancement of *LacZ* Expression in AdCEALacZ-infected LoVo Cells by Addition of IL-6

Addition of IL-6 had no remarkable effect on the internal (background) *lacZ* activity of the LoVo or HeLa cells. Interestingly, the AdCEALacZ-mediated *LacZ* activity in IL-6-pretreated LoVo cells was approximately 40% higher when compared with AdCEALacZ-infected LoVo cells without IL-6 pretreatment. In contrast, the addition of IL-6 did not enhance the *lacZ* activity in AdCEALacZ-infected HeLa cells.

DISCUSSION

Strategies of gene therapy for cancer include the delivery of therapeutic genes to enhance the antigenicity of tumor cells as vaccine by inducing cytokine genes[29,57], to alter the malignant phenotypes by targeting dominant oncogenes and mutated tumor suppressor genes[14,24,43,53,65], and to convey cell death by transducing lethal or suicide

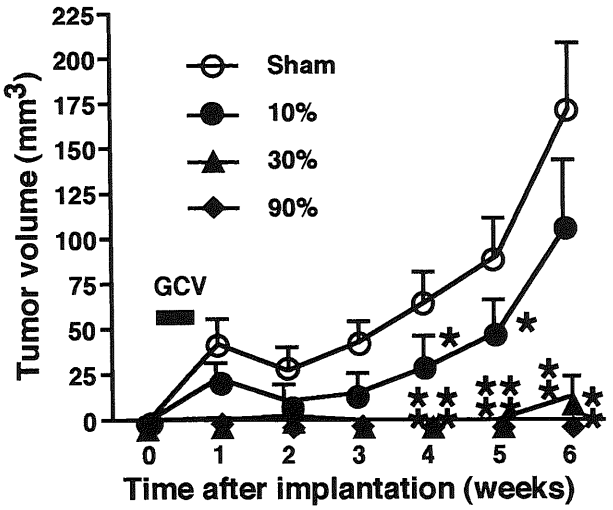

Fig. 7. Suppression of tumor growth by *in vivo* bystander effect.
LoVo cells infected with AdCEATK at moi 10 were washed with minimal essential medium containing 10% heat-inactivated FCS and mixed with sham-infected cells at various ratios (infected cells:sham-infected cells = 0:10, 1:9, 3:7 or 9:1). Then the cells were implanted to nude mice (n=7 for each ratio) followed by GCV administration, and the tumor volume was estimated as detailed in the methods.
*, **:Statistical significance compared with tumors of sham-infected cells (*:$p<0.05$; **:$p<0.01$) through Kruskal-Wallis test followed by Fisher's protected least significance difference procedure.

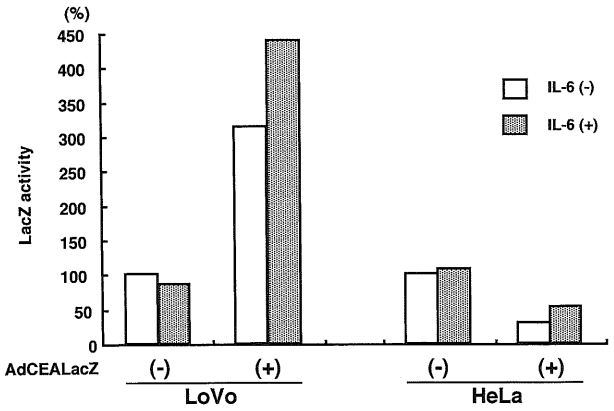

Fig. 8. *LacZ* activity in AdCEALacZ-infected LoVo and HeLa cells with (dotted bar) or without (open bar) addition of IL-6 *in vitro*.
The cells were pre-incubated in medium with or without IL-6 for 5 days, then infected with AdCEALacZ. After incubation for 72 hr, quantitative analysis of *LacZ* expression was performed by FACS-Gal analysis.
Enhancement of *LacZ* expression in AdCEALacZ-infected LoVo cells was observed on addition of IL-6.

genes[5,10,19,26,38,47,52,55,58]. Among them, utilizing suicide genes seems to have a chronological advantage in that the cytotoxicity can be regulated at two different steps: at gene delivery and prodrug administration. With the suicide gene strategy, the most appropriate time to start therapy is selected depending on the condition of the patient. In addition to this chronological advantage, regulation as a function of space, the cell type-specific expression of transgenes, has been investigated as a means of higher efficacy and safety in gene therapy[10,26,38,42,51,58]. Besides the target cell-specific delivery to reduce the toxicity to non-malignant cells of the host, the suicide gene strategies require transcriptionally targeted vectors that can restrict the expression of therapeutic genes to appropriate cells.

Based on the fact that a significant number of cases with colorectal and lung cancer bear CEA-producing adenocarcinomas[16,34,40,63,64], and the knowledge regarding the transcriptional regulation of CEA gene elucidated by others[41,50], CEA was chosen as a model gene to test the feasibility of cell type-specific expression. Although the mechanisms of the upregulated expression of CEA gene in adenocarcinoma cells are not fully understood, putative mechanisms are thought to include hypomethylation in the upstream of the gene[60], *cis*-acting elements[42,51], and further distant 5' or 3' elements[42] e.g. the intronic regulatory region in CEA family genes[49]. Due to the limited room available for foreign DNA in the genome of the first generation adenovirus vectors[31], our trial was to deliver a short DNA fragment containing the *cis*-elements conveying cell type-specific expression to CEA-producing cells. When infected with AdCEALacZ, CEA-producing LoVo, VMRC-LCD, and VMRC-LCR cells showed a significantly higher *lacZ* expression compared with CEA-non-producing cervical cancer HeLa and adenocarcinoma ABC cells, as observed in gastric cancer cell lines in previous reports[26,57] indicating the ability of the *cis*-elements of the CEA gene to confer restricted expression of a heterologous gene. However, although Osaki et al have reported the usefulness of the CEA promoter sequence -322 to +111 in A549[38], there was no favorable *lacZ* expression in A549 cells infected with our AdCEALacZ, indicating that this CEA promoter is not "universal" for CEA-producing cells. In CEA-producing malignancies in which this CEA promoter does not show favorable promoter activity, mechanisms other than the *cis*-elements contained in the promoter sequence play important roles in the expression of CEA. To achieve transcriptionally targeted expression in such cells, a more distant and long regulatory sequence of CEA gene[42] should be tested in a new generation of adenovirus vectors that have room for longer foreign DNA by deletion of viral genomic DNA leaving only an inverted terminal repeat[25], or upregulation of CEA expression using some cytokines (e.g. IL-6).

With AdCEATK, a greater *in vitro* GCV sensitivity was transduced in LoVo and VMRC-LCD than in HeLa and ABC cells, and LoVo cells infected

with AdCEATK formed no tumors, whereas AdCEATK infected HeLa cells developed into apparent tumors, indicating that transcriptionally targeted killing of CEA-producing cells was possible by reducing the collateral expression of HSV-TK gene and hence the toxicity to CEA-non-producing cells. Since the mechanisms of upregulated expression of CEA gene in malignancies vary among individual cases and sensitivity to the HSV-TK/GCV system varies among CEA-producing malignancies, clinical use of the present strategy in the future should be based on *in vitro* evaluation of the promoter activity and the HSV-TK/GCV sensitivity of tumor cells in individual cases. In our study, as could be expected from the result of *in vitro* sensitization to GCV, *in vivo* transcriptionally targeted killing was reproducible in nude mice. Thus, by targeting appropriate tumors, transcriptional targeting should be feasible.

Because the tools available today are imperfect in terms of the efficacy of gene delivery even *in vitro*, *in vivo* gene transfer is much more challenging. Suicide gene transfer using the vectors available at present would leave many tumor cells that should be killed without direct transduction of the gene. A promising *in vivo* bystander effect was observed in the present study. *In vivo* tumor growth suppression of LoVo cells pre-infected with AdCEATK was more remarkable than expected from *in vitro* GCV sensitivity. Further, pre-infection of 10% of the LoVo cells comprising tumors resulted in significant tumor growth suppression, and the pre-infection of 30% of the LoVo cells caused a total elimination of tumors. The efficacy of the bystander effect is known to vary widely depending on cell type[11,30,32]. The *in vitro* bystander effect of the HSV-TK/GCV system is thought to require the passage of GCV-triphosphate through gap junctions between adjacent cells[9,12,32]. In regard to *in vivo* effects, bystander killing using the HSV-TK/GCV system was reported in B cells lacking gap junctions[45], suggesting that unknown mechanisms including close packing of tumor cells leading to the uptake of apoptotic vesicles[20], immune cells other than T lymphocytes such as macrophages and natural killer cells[1,15], and blood vessel destruction potentially contribute to the killing of uninfected tumor cells. These findings indicate that tumors could be totally eliminated *in vivo* without serious toxicity to the hosts as long as appropriate targets were chosen by *in vitro* evaluation and the therapeutic genes were delivered to a sufficient number of tumor cells. Based on previous studies by others[6-8] reporting upregulated surface CEA expression of IL-6 treated CEA-producing cells, we evaluated the enhancement of cell-type specific CEA promoter-driven gene expression. Addition of IL-6 enhanced AdCEALacZ-mediated *LacZ* activity in CEA-producing cells, suggesting the possibility of raising the efficacy of the cell-type-specific gene therapy using tissue specific promoter. In future, the achievement of enhanced cell-type specific toxicity with a reduced toxicity to normal tissues should be pursued further. For this, the combination of several cell-type specific strategies such as simultaneous usage of cell type-specific promoter–driven vectors (e.g. CEA and midkine[33]) and the Cre/LoxP system[41] might be candidates.

One of the most clinically convenient ways to deliver an anticancer reagent to patients is intravenous administration as long as the toxicity is restricted to cancer cells. Theoretically, either by specific delivery or transcriptional targeting, the tissue-specific vectors are ideal for systemic administration. Since intravenous injection of mice with adenovirus vectors leads to preferential transduction of transgenes to liver tissue [3,39], multiple liver metastasis of CEA-producing adenocarcinoma including colorectal and lung cancer might be the possible target for systemic administration of transcriptionally targeted vectors. But since liver function damage, due to the infection of adenovirus vectors, has been observed[54,62], careful attention and observation is needed when using adenovirus vectors.

In conclusion, this study has shown that adenovirus vectors containing a CEA promoter convey a preferential expression of the heterogous genes in CEA-producing colon and lung cancer cell lines and that cell type-specific *in vivo* tumor growth suppression is possible when a suitable target is selected by *in vitro* evaluation. Thus we have demonstrated the feasibility of CEA promoter-driven cell-type-specific killing *in vitro* and *in vivo*. These findings should encourage further improvement of the promoter to enhance cell-type-specific expression as well as of the method for administration of the vector for future clinical application of this strategy.

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